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Several small animal models of HIV infection have been proposed. We have developed a simple means to support the replication of HIV-1 which can produce large populations of infected mice for therapy screening and host-virus interactions.

Nude mice (3-4 wks old) were exposed to 600 R's of ^{137}C's irradiation and inoculated with 1 x 10^7 HIV-1 infected CEM cells. The animals were followed daily for weight gain and tumor progression. Groups of 6 animals were exsanguinated at 8 intervals over a 9 week period and necropsies were performed.

Plasma p24 antigen was detected at day 3 and rose over 9 weeks (2200 pg/ml). Where possible, plasma antigen was neutralized with human antisera to HIV-1. The infected mice did not exhibit any weight loss, but a highly significant difference was seen in tumor progression when compared to controls. Using immunohistochemistry, sheep raised polyclonal antibodies to viral p24 and gp 120 detected HIV proteins within tumor cells and mouse splenic macrophages.

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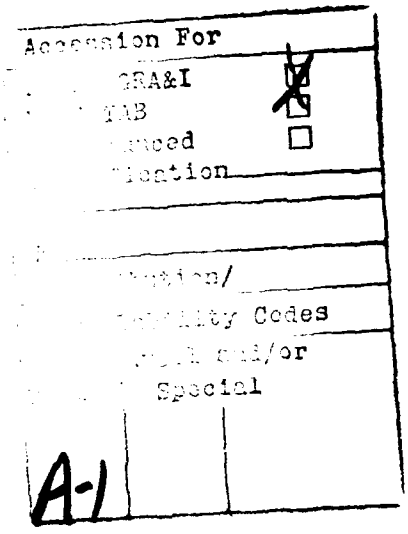
19. Abstract continued:

Electron microscopy revealed rare intracellular lentivirus particles in the CEM cells.

These findings suggest that the nude mouse transplanted with a well characterized HIV permissive cell line should be useful for many immunological and antiviral studies.



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_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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INTRODUCTION

I. NATURE OF PROBLEM

It is now widely accepted that the disease termed Acquired Immunodeficiency Syndrome or AIDS is caused by the human immunodeficiency virus (HIV-1) (1). Since AIDS is hallmarked by the appearance of opportunistic infections, this classification represents the final stage (Walter Reed -6) of a long-term lentivirus infection. Recently two other related human (HiV-2) and primate (SIV) lentiviruses have been identified and sequenced (2,3). Although HIV-2 produces disease similar to HIV-1, it is less prevalent worldwide (4). At present there is no vaccine available for the prevention of HIV disease. The best therapeutic agent against the disease is azidothymidine (AZT), the only FDA approved anti-HIV. This inhibitor of viral reverse transcriptase has been shown to be beneficial in increasing the quality of and prolonging the life of infected individuals by reducing the incidence of opportunistic infections, by increasing the number of CD4+ cells (5), and by reducing the levels of circulating p24 gag antigen (6). This drug has several drawbacks, most notably bone marrow suppression in 25% of treated patients. AZT has also been shown to be much less inhibitory for HIV-2 *in vitro* (7). Fortunately, due to the unique genome of these lentiviruses (8), several approaches are available to develop therapeutic strategies which exploit this uniqueness. Currently many compounds exist which are targeted to interfere with the expression of HIV genes (Dr. Michelle Vinocour,NIAID, personal communication). So far, clinical trials in humans infected with HIV-1 have been disappointing for most early generation compounds, although some benefit has been seen with Foscarnet (a DNA polymerase inhibitor) and interferon (9). Although HIV-2 can infect the Rhesus monkey (10), and HIV-1, until recently, could only infect the human, chimpanzee, and gibbon, attempts at the direct infection of small animals have been unsuccessful (11), leaving *in vitro* assays and controversial clinical trials (12) as

the only means to test antivirals and host cell-HIV interaction. Beyond the obvious need to test potential therapeutic agents, singly or in combination, animal models of HIV disease are needed to study several facets of the infection (Table I).

TABLE I

ASPECTS OF HIV DISEASE THAT CAN BE ASSESSED BY ANIMAL MODELS

	Area of Study	Reference
1.	Vaccine development	Koff (13)
2.	Cellular immune response	Krowka (14)
3.	Humoral immune response	Krowka (14)
4.	Viral and cellular determinants of infection	Levy (15)
5.	HIV strain variation (molecular biology)	Sakai (16)
6.	Viral adaptation to other species	Fields (17)
7.	HIV pathogenesis	Kurth, et al, (10)
8.	Factors that determine the course from infection to disease	Levy (15)
9.	Transmission Horizontally (between given species) Vertically (to progeny)	Kurth, et al, (10)
10.	Development of improved diagnostic and prognostic assays	Kemp, et al, (18)

II. BACKGROUND OF PREVIOUS WORK

Animal models of HIV infection can conveniently be placed into four categories (19): (one) immunodeficiency inducing retroviruses not closely related to HIV, (two) lentiviruses of non-primate species, (three) lentiviruses of non-human primates,and (four) HIV infection of non-human species. Each of these categories offers an approach to study some aspect of HIV disease.

Although the Raucher Murine Leukemia Virus (RMuLV) has been used to screen antivirals (20), the other viruses from category one, e.g , Friend Murine

Leukemia Virus (FMuLV), Feline Leukemia Virus (FeLV), and the Macaque Type D SAIDS retrovirus (SRV) have been used to mimic the late stages of HIV infection, most notably immunosuppressive disorders such as opportunistic infections and wasting syndrome (21). The lentiviruses of non-primate species, Maedi-Visna Virus of sheep (MVV), Caprine Arthritis-encephalitis Virus (CAEV), Equine Infectious Leukemia Virus (EIAV), and Bovine Leukemia Virus (BLV), have been most useful to study the pathogenesis of lentivirus infection, particularly virus/host cell interaction, immunopathogenesis, and neuropathology. Additionally these viruses have been used to investigate the factors which trigger the transition from latent virus infection to productive and lytic cycle and the molecular biology of hypervariable env gene sites which are similar to those seen in HIV (10). One other animal lentivirus, Feline Immunodeficiency Virus (FIV), has had some utility in the study of protective immunity and in screening antiviral agents (10).

The lentiviruses of non-human primates are represented by the pathogen most closely related to HIV-2, the simian immunodeficiency virus (SIV) of which seven different strains have been identified, being isolated from different hosts: Rhesus monkey (SIVmac), stump-tailed macaque (SIVstm), pigtailed macaque (SIVptm), Cynomolgus monkey (SIVcyn), sooty mangabey (SIVsm), African green monkey (SIVagm), and the mandrill (SIVmnd). Of the seven at least two (SIVmac and SIVsm) readily infect human cells *in vitro* (10). Although primates must be used, the SIV- infected animal appears to be the one model which most closely mimics the human disease, and therefore has the greatest utility for vaccine trials (13).

HIV-1 infection (defined as when whole virus or viral protein products can repeatedly be isolated and are usually associated with a detectable immune response after a single inoculation of virus) of nonhuman species has been attempted and is apparently successful in chimpanzees, gibbons, apes, rabbits, and mice. As it might be expected, HIV-2 appears to have a wider range of species

susceptibility with early indication of transmission to Rhesus monkey and baboons (22). It is generally agreed that the primates, most notably the chimpanzee, can be used only as an endpoint and not for the screening of experimental vaccines and therapeutic agents. Therefore, it is imperative that common laboratory animals such as the rabbit and mouse be used as *in vivo* models for HIV experimentation.

Since HIV-1 is not readily transmissable to common laboratory animals (11), novel systems had to be developed (23). All of these systems require some manipulation of the host and/or of transplanted cells resulting in inherent disadvantages which must be balanced against the expected utility of each system. Three systems, one reported and two potential, are available for the infection of HIV-1 in lagomorphs. Varnier's laboratory produces aseptic thioglycollate peritonitis in small numbers of rabbits, followed by intraperitoneal (ip) injection of H9 cells infected with HIV. The rabbits produce an antibody response to the HIV for several months in response to viral antigenemia. This model may have some utility for vaccine development; however, few animals are produced since the peritonitis causes a high degree of mortality and morbidity (Dr. Nick Ellis, Burroughs-Wellcome Laboratories, personal communication). The first report of infecting rabbits with human virus occurred in Japan when HTLV-I was transmitted to rabbits by transfusion. The infected blood was serially passaged five times and vertical transmission occurred from dam to offspring via milk (25). Based upon these findings, and previous *in vitro* results that rabbit cells can be infected with HIV-1 with prior simian virus 40 (SV40) or HTLV-I infection (26), Kulaga et al attempted the HIV-1 infection of rabbits. HTLV-I/HIV-I challenge resulted in antibody production and variable clinical disease (diarrhea and weight loss). This most recent report (27) also concludes that prior HTLV-I infection is not required to infect rabbits. Transplantation of human HIV-1 producing cells (A3.01) alone can produce antibodies (at low levels), and some disease symptoms. This approach should

provide utility to study several aspects of HIV disease. The finding that lagomorphs can be infected with HIV-1 offers encouragement that rodents can also be infected with appropriate manipulation.

It is clear that rabbits infected with HIV-1 offer much for HIV *in vivo* experimentation due to the large volume of serum and cells available for testing, and this species would provide a logical prelude to experimentation on all primates (including Man). However for large scale studies where statistically significant numbers of animals are needed, the mouse is ideal. In addition, through germline manipulation, either through crossbreeding or ovum microinjection, strains of mice are available which can mimic HIV disease. Table II lists all potential mouse systems to date which have been suggested as AIDS/HIV disease models and the aspects of the disease that a given model can address.

As discussed earlier, the MuLV's have some utility in addressing immunodeficiency and assessing antiretrovirals but are limited in scope by the viral genome and neoplastic disease. The microencapsulation assay (28) was originally designed to assay the *in vivo* effects of antineoplastic agents (29), and has now been extended to include antivirals. Although the utility extends only 10 days and the drug level within the capsule is lower than the plasma level, it has been presented that HIV infected CEM cells within the capsule demonstrate less HIV replication in AZT-treated mice than untreated controls after 10 days (30). The construction of transgenic mice containing HIV proviral DNA was recently reported (31). This model of HIV latency produces Founder (F_0) animals with full length proviral DNA integrated into their genome but without any observable ill effects. The offspring (F_1) of a single dam mated with nontransgenic males produced several pups which lived up to 25 days. These pups displayed marked epidermal hyperplasia, lymphadenopathy, splenomegaly, pulmonary lymphoid infiltration, growth retardation, and infectious HIV. These animals appear to be most useful to study

TABLE II. PROPOSED MURINE MODELS OF HIV DISEASE PROPOSED MURINE MODELS OF HIV DISEASE

	Ref. No.	HIV +	Virus Caused Immuno-suppression	Virus Caused Pathogenesis	Circulating HIV	Immune Response to HIV	Mouse Cells HIV +	Available for Large Numbers	Long-term or Short-term Study	HIV Molecular Biology
1 Friend MuLV C57BL/6	21	-	+	+	-	-	-	+	short	-
2 Raucher MuLV BALB/c	20	-	+	+	-	-	-	+	short	-
3 Microencapsulated HIV infected cells CD-1	29,30	+	-	-	-	-	-	+	10 days	-
4 Transgenic HIV Provirus	31	+	F_1 animals only	F_1 animals only	F_1 animals only	F_1 animals only	+	No	25 days	+
5 SCID-hu	37	+	-	-	-	-	-	?	2 months +	+
6 SCID-human EBV + PBL	36	(1)								
7 bg/nu/xid human bone marrow graft	32	(1)								
8 xenotransplated HIV + cells nude/immuno-suppressed mice	39	+	-	(2)	+	+	+	+	2 months +	+

(1) Potential, not yet reported or presented
(2) Understudy

the pathogenesis of HIV disease, however the small numbers produced may limit their widespread utility.

Only within the last quarter of 1988 has the use of immune deficient mice emerged as the most promising murine model of HIV infection. Kamel Reed and Dick (32) have recently reported a new application for a previously little used strain of mouse and a strategy to engraft human hematopoietic stem cells into mice which successfully produce large numbers of human granulocyte macrophage colony/forming units (hCFU-GM). These bg/nu/xid mice have low numbers of natural killer (NK) and lymphocyte-activated killer (LAK) cells, but require the constant infusion of human IL-3 and GM-CSF plus gamma irradiation (400 cGy) to achieve a successful engraftment. These animals have not yet been challenged with HIV but it appears that they should have the potential to support viral replication. The severe combine immunodeficient (SCID) mice were characterized several years ago at the Fox Chase Cancer Center (33). This mutant lacks functional T and B lymphocytes leading to premature death (even when housed under specific pathogen-free [SPF] conditions) and a high incidence of spontaneous T-cell lymphomas (34). Unlike the bg/nu/xid mice, SCID mice apparently have high levels of NK cells (35). Mosier, et al. (36) successfully transferred a functional human immune system to SCID mice. The xenogeneic transplant of human peripheral leukocytes was accomplished by intraperitoneal (ip) transfer of cells from Epstein-Barr Virus (EBV) seropositive donors. These mice exhibited a human antibody response, transient circulating human T-cells, and sporadic development of human EBV + B-cell lymphomas. Like the Kamel-Reed and Dick model this system has not yet been challenged with HIV, but appears to have the potential to support viral replication. The first incidence of SCID mice being infected by HIV-1 was recently reported by Namikawa et al. (37). This group previously reported (38) that SCID mouse colonies can be maintained for extended periods by the continual administration of

prophylactic trimethoprimsulfamethoxasole (TMS) in the drinking water. This agent suppresses *Pneumocystis carinii*, the opportunistic pathogen most common in SCID mice. A hematochimeric SCID-hu mouse was created by the engraftment of human fetal liver, thymus, and lymph nodes. The human hematopoietic cells of the T and B cell lineages were observed to differentiate, but while phenotypically mature human T-cells are found in peripheral blood, their appearance is time-dependent and time-limited. Since mature human T-cells were produced in the SCID mice, direct HIV-1 challenge of the transplanted human fetal thymus or lymph node was attempted. The animals were followed for eight weeks and the human tissue was monitored by immunohistochemistry and *in situ* hybridization using a HIV-1 specific antibody and probe. Semi-quantitation of viral expression was determined by light microscopy which revealed increasing numbers of human cells expressing HIV viral genes. This expression was noted more often in the SCID-hu thymic medulla than in the thymic cortex. This model of HIV infection should aid in the *in vivo* study of HIV infection at the molecular and cellular levels, but until better means of viral quantitation are possible within the system, many other aspects of the model cannot be assessed (see Table II).

III. PURPOSE OF PRESENT WORK

The first presentation of the replication of HIV-1 in immunosuppressed mice was presented before the Twenty-eighth Interscience Conference on Antimicrobial Agents and Chemotherapy in October of 1988 and was funded under this contract (39). It was the goal of this work to present a mouse model capable of supporting HIV-1 replication; this having been achieved, it is the current goal of this work to utilize this data to develop a functional and usable mouse model of HIV infection.

The first in-depth description of an immunosuppressed mouse was the nude (nu) mutation decribed by Flanigan in 1966 (40). Due to the short lifespan of the animals (25 weeks), little interest in this mutant was generated until Pantelouris (41)

reported the significant finding that only a rudiment of a thymus could be found in nudes of any age. The mutation is inherited as an autosomal recessive. Offspring are produced by mating homozygous males with heterozygous females, yielding 25% homozygous males and 25% homozygous females (42).

Believing correctly that these animals had impaired cell mediated immunity, Rygaard (43) subsequently demonstrated the diminished rejection phenomenon by the successful grafting of rat skin onto these immunodeficient animals. Further successful attempts by Rygaard and Povlson (44) at xenotransplantation of human malignancies extended the usefulness of these mice.

As knowledge about the "nudes" increased, it appeared that the life expectancy could be extended to a near normal level by housing the animals in strict specific pathogen free (SPF) conditions. Wasting disease, common among neonates, and communicable infections were all but eliminated (42). Under these conditions, the potential applications of the nudes could be expanded.

Initially, one finding was disturbing. It was observed that many xenotransplants did not grow in the nudes, particularly neoplasms of hematopoietic origin, in spite of apparent optimum conditions. While it was classically known that the immunological protection mediated through the T-cell was diminished or nonexistent, several investigators (45, 46) identified a previously unrecognized null cell involved in tumor rejection which is higher in nu/nu mice than any other mouse strain. Since prior antigenic sensitization was not required to reject foreign tissue, it was designated the natural killer (NK) cell. Activity of these cells is highest when the animal is five to ten weeks of age. Younger and older animals have notably lower reactivity (47). More recent work shows convincingly that NK cells have no effect on some transplanted human tumors, most notably CEM cells (35). Lymphokine-activated killer (LAK) cells appear to be an additional immunologically responsive cell found in mice with a nu mutation only. These cells when activated with

interleukin-2 are broadly lytic for fresh tumor cells *in vitro*. Since many human solid tumors grow readily after heterotransplant into nude mice, this animal continues to be used as an *in vivo* model to assess the susceptibility of malignancies to chemotherapy (48, 49), radiotherapy (50), immunotherapy (51),hormonal manipulation (52, 53), or biological response modifiers (54, 55) either alone, or in combination.

The acquisition of new virus-associated antigens by tumor cells through infection with viruses and subsequent recognition of the new antigens as foreign by the tumor-bearing host has been known (56). This process of "viral xenogenization" has been suggested for use as a biological response modifier to control neoplastic growth (57). In an attempt to test the effects of viral xenogenization on heterotransplanted tumors, Reid et al. (58) transplanted RNA mumps virus infected human HeLa cells, or RNA vesicular stomatitis virus-infected hamster BHK cells into nude mice. Tumors were produced, but at decreased size and incidence when infected with either virus, suggesting a host immune response. More importantly for our purposes, infectious viral particles were found only when the mice were given 500 total body rads of ^{137}Cesium. These results give a firm foundation that 1) human viral permissive cells can proliferate in athymic mice and 2) that RNA viruses capable of growing in permissive cells can produce complete (and presumably infectious) virus in hosts not normally associated as recipients. Although the natural history of the virus is artificial, the virus production *in vivo* is not.

Using the nude mouse to support the growth of HIV permissive human lymphoid cells, we believe we have an animal model in which we can investigate tissue changes, gain insight into the replicative cycle of the HIV, and study antigenemia. Potentially, we will be able to measure *in vivo* effects and toxicity of various anti-HIV treatments. Our proposed model will not enable us to adequately

study immunosuppression, since these animals are already athymic, but it is our thesis that the best treatment for HIV disease is before gross immunosuppression and secondary disorders occur.

IV. METHODS OF APPROACH

The overall approach for the first half of the contract period was to determine if the nude mouse heterotransplanted with a human HIV-permissive cell line was capable of supporting HIV replication and to refine established techniques (laboratory tests) to assess the replicative cycle of HIV.

Our approach consisted of an initial phase where the dynamics of uninfected CEM transplants were determined, and the effect of the tumor on the mouse was assessed. Specifically, tumor size and growth kinetics, animal morbidity (as assessed by the ability of the animal to thrive), and the role of gamma irradiation in tumor establishment were determined. After these baselines were established, we challenged tumor bearing mice with HIV-infected CEM cells. The effect of the virus on baselines was assessed and virus was detected both in tumor tissue (through nucleic acid analysis and immunohistochemistry) and in plasma (through p24 antigen assay).

The next approach taken was that of determining if an initial inoculum consisting of both infected and non-infected cells (thereby providing uninfected target cells in the inoculum) would result in HIV replication in the mouse and if there would be any effect on the baselines established earlier. Various ratios of infected to non-infected cells were used, including 100% infected. Virus was detected as above.

Concurrently with the HIV-infected cell studies, the effect of antigen levels in the tissue cultures used to infect the animals was assessed. Presently, we are determining viral distribution in highly antigenemic mice through extensive

histopathology as well as plotting antigenemia curves from 3 days through nine weeks *in vivo*.

In collaboration with Dr. Barney Graham, preliminary attempts at establishment of CEM transplants in neonatal BALB/c mice were made and the successful HIV challenge of human HIV-permissive heterotransplants in immunosuppressed adult BALB/c adult mice accomplished.

Initial studies utilizing patient material involve PHA-stimulation of lymphocytes. Antigen levels of 3-5 day supernatants of stimulated cells were measured. The cells, in combination with uninfected CEM cells, were inoculated into irradiated nude mice. Ten days post tumor onset, the animals were sacrificed and plasma was assayed for p24 antigen.

BODY

I. EXPERIMENTAL METHODS

A. Virus. HIV-1 (HTLV-IIIB strain) was originally obtained from the laboratory of Dr. Robert C. Gallo (NCI) in1984, and has been maintained in the BL-3 facilities of Vanderbilt University's Department of Pathology since its acquisition. Infectivity assays are routinely performed using the method of Montefiori et al (59).

B. Cells. CCRF-CEM cells (60) were obtained from the laboratory of Dr. L. Montagnier (Pasteur Institute) in 1984. A sub clone of this culture was confirmed as CCRF-CEM by cytogenetic analysis (Dr. Ward Peterson, personal communication,1985), and has been maintained in RPMI-1640 with 15-20% fetal calf serum at pH 7.2-7.4 at 37°C. The monocytic cell line U937 was obtained from the American Type Culture Collection (ATCC CRL 1593) and maintained in a similar manner as CCRF-CEM cells.

C. Animals, Diets, and Environment. Outbred, athymic, 3-4 week old nude (nu/nu) mice were purchased from Harlan Sprague Dawley, Inc., and housed at

27° +/-1° without antibiotic coverage in a specific pathogen-free room under laminar-flow HEPA-filtered air. All bedding, cages, water, and other material coming in direct contact with the animals are autoclaved before use. Animals are permitted access to food and water ad libitum. The mice are fed a diet of pelleted chow (Wayne Sterilizable Lab-Blox, Allied Mills, Inc., Chicago,Il.), which contains elevated levels of heat-sensitive nutrients. The cages were covered with bonnets and otherwise handled as outlined under BL-3 conditions, as described by NIH guidelines. When irradiated,mice were exposed to 609 +/-15 Roentgens (R) of gamma radiation at the body surface (determined by thermal luminescent dosimetry TLD) using a ^{137}Cs Mark I irradiator (JL Shepherd and Assoc, Glendale, CA.).

D. Heterotransplantation, Animal Monitoring, and Necropsy. Cell cultures from suspension were washed in serum-free RPMI 1640 and harvested. Inocula were standardized by counting the viable cells with a hemocytometer and adjusting the cell suspension with serum-free medium for an inoculum volume of 0.2 ml. Non-infected cells were injected subcutaneously into the intrascapular region using a 3 cc syringe with a 23 gauge needle. Inoculum leakage is diminished by tunneling through the tissues. To avoid the use of needles when injecting HIV-1 infected cells, a 22 gauge teflon coated catheter was inserted as described above, the needle removed, and a syringe luerlocked to the catheter. Animals were observed at least 5 days a week until termination of experiment. Tumor onset was determined by palpation of inoculation site daily and tumor size in two dimensions is monitored via calipers. Tumor volume was calculated from the formula for a prolate elipsoid $\pi/6\ LW^2$ where L is the long dimension and W is the short. Animals inoculated with HIV were observed for any behavioral changes. Animals were anesthesized with a ketamine/xylazine mixture and exsanguinated after each experiment before tumor burden was stressful. The primary tumor is removed within minutes after death and

representative samples of brain, lung,liver, spleen, and kidney were frozen or fixed in 4% buffered formaldehyde, 2% glutaraldehyde, or cold acetone.

E. HIV Detection Techniques. Circulating HIV antigen was measured using Abbott Diagnostics' Antibody to HTLV-III Antigen EIA, which detects the p24 gag protein of HIV. Specifically, the assay consists of polystyrene beads coated with human antibody to HTLV-III which are incubated at room temperature overnight with 200 µl of test plasma or tissue culture supernatant. The rest of the "sandwich" consists of rabbit polyclonal antibodies to HTLV-III and peroxidase conjugated goat anti-rabbit IgG. Results are calculated from standard curves run with each test and converted to pg/ml of sample. When possible, reactive samples were confirmed using Abbott Diagnostics' Antibody to HIV Antigen Neutralization Test which consists of pooled human neutralizing antibodies to HIV. Normal mouse plasma was run as negative control, in addition to the negative human control in the test kit. No normal mouse plasma was ever found to be reactive; the background reading with murine plasma was consistently found to be less than the human negative plasma background. Details of the assay have been published previously (61). The presence of HIV in tissues was determined through nucleic acid analysis and immunohistochemical staining of plastic embedded sections. RNA was extracted from frozen tissue sections by the method of Chirgwin, et al (62). Briefly, tissue is homogenized in 4.5 M guanidinium buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 2% sarkosyl, 1% mercaptoethanol and the homogenate layered on a 5.7 M CsCl, 50 mM EDTA cushion and centrifuged for 5 hr. in a Beckman Ti 75 rotor at 55K. RNA pelleted at the bottom of the centrifuge tube is dissolved in buffer, phenol/chloroform extracted and precipitated in ethanol. DNA is recovered from frozen tissues by digestion in 10 mM Tris, pH 8.0, with 75 mM NaCl 20 mM EDTA, and 0.2 % sarkosyl with proteinase K (100 µg mg/ml) at 37° until completion, then phenol/chloroform extracted and ethanol precipitated. Purity is checked and

quantitation determined by UV absorbance values; 230 nm/260 nm value < .50 and 260 nm/280 nm values of 1.80 for DNA and 1.90-2.00 for RNA were routinely achieved. Quantitation is calculated from the 260 nm reading, with an E1% cm of 250 for RNA and an E1% cm of 200 for DNA.

Extracted nucleic acids were tested for the presence of HIV proviral sequences or HIV message by Southern or northern analysis. For Southern analysis, 10 µg of DNA was digested overnight with restriction endonucleases and fractionated on a 20 cm 1% agarose gel and then transferred to positively charged nylon membrane (BioRad Laboratories) using the procedure of Southern (63). Total cellular RNA (20 µg) was fractionated on a 1.2% agarose gel after being denatured in glyoxal/DMSO following the procedure of Maniatis (64). Transfer to nylon membrane in alkaline buffer was according to BioRad procedure for alkaline northern blot (65). Detection of both provirus and viral message was through hybridization with 32P labelled nick translated DNA HIV sequences (66). The probe used was isolated in our laboratory from pBH10-R3 (67) plasmid containing proviral sequences of HTLV-IIIB minus the LTR's. Plasmid purification was by the alkaline hydrolysis method with separation on a CsCl gradient following Maniatis (64). The specific sequences used for nick translation were isolated by restriction endonuclease digestion of the plasmid at the Sst I and BamHI sites resulting in the 1.3 Kb 3' nef fragment which was separated on an agarose gel and purified using the Geneclean system (Bio 101, La Jolla,CA). Acetone fixed tissues were embedded in plastic embedding medium (JB4, Polysciences, Warrington, PA) and sectioned at 2 microns, and stained for HIV gp120 or p24 using the method of Casey, et al (68). Specific antibodies used were sheep polyclonal anti-human gp120 and anti-p24 (Accurate Corp., Westbury, NY) and rabbit anti p24 (Abbott Laboratories, Chicago) primary antibodies, rabbit anti-sheep secondary antibody (Dako Corporation, Santa Barbara, CA), and peroxidase conjugated swine anti-rabbit immunoglobulins (Dako Corp.) tertiary antibody.

When necessary, tissue sections were quenched prior to primary antibody incubation in 0.1% sodium azide/ 0.3% hydrogen peroxide solution. Two per cent normal swine serum was used as a blocking agent. Diaminobenzidine (Sigma Chemical Company, St. Louis, Mo) was used as the reaction substrate.

Glutaraldehyde fixed tissue was processed for transmission electron microscopy after fixation for 1 hr. at 4 C in 2.5% glutaraldehyde/ 0.1 M cacodylate buffer by washing in cacodylate buffer, post'fixing in 2% OsO4 for 1 hr., and staining with 1% aqueous uranyl acetate. After graded ethanol dehydration, the tissue was stained with toluidine blue, infiltrated with ethanol/EPON for 1 hr. and embedded in EPON. The EPON is cured for 12 hrs. at 55 C, the elastic partially polymerized EPON layer peeled off and further cured for 24 hrs. at 60 C. Thin sections (500 Å) were cut and stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope for HIV particles.

F. Statistical Analysis. Statistical analysis was done using a two-tailed Student's T-test for unpaired data. Significance was determined at the $p = .05$ level, with marginal significance being defined as $p > .05$ but $< .10$.

G. PHA Stimulation of Lymphocytes. Mononuclear cells were separated from 20 mls blood collected in heparinized tubes on Ficoll-hypaque gradients washed in serum-free RPMI-1640 medium, and resuspended in 5 mls. RPMI with 20% fetal calf serum containing 10 ug/ml lectin (Sigma Chemical Company). Cells were then cultured for 3-5 days, centrifuged and the supernatant checked for p24 antigen using the Abbott EIA. The cells were washed with serum-free RPMI and injected along with uninfected CEM cells into irradiated nude mice.

II. RESULTS OBTAINED

A. Growth of CEM Cells in Nude Mice Duplicate populations ($n = 6$) of athymic (nude) mice were inoculated with 0.5, 1, 2, or 5 X 10.7 CEM cells. One group

of each inoculum was irradiated one day prior to cell inoculation. Daily weights and tumor measurements were taken. Comparison at each inoculum between irradiated and non-irradiated animals showed significant differences in mean tumor volume at all levels starting 5-7 days post onset (Figure 1). Among irradiated groups differences are shown in Table III. No stastical differences were found in weight gain between groups (Figure 2).

TABLE III

INOCULA	RESULTS
0.5×10^7 vs 1.0×10^7	**Significant 10 d post-onset to termination1**
0.5×10^7vs 2.0×10^7	***Transient marginal differences (d 23,30,33)***
0.5×10^7vs 5.0×10^7	**Significant 10d post-onset to termination**
1.0×10^7vs 2.0×10^7	**No significant differences**
1.0×10^7vs 5.0×10^7	**Significant 14d post-onset to termination**
2.0×10^7vs 5.0×10^7	**Significant 14d post-onset to termination**

B. HIV-I Challenge of Established CEM Tumors. In order to determine if established CEM tumors can support the replication of HIV-1, four populations of CEM-bearing mice were challenged 11 days after CEM transplantation with either uninfected (control) or one of three differing inocula infected with HIV-1. This experiment was abruptly terminated when it was discovered that the mice were delivered infected with murine hepatitis virus. There have been no problems with this virus since that time.

This basic approach was repeated. Twenty-four animals were inoculated with 0.5 X 10^7 CEM cells. On day 13 when all animals were positive for tumor onset, three populations of six animals each were challenged with either $1X10^4$, $1X10^5$, or $1X10^6$ 100% HIV-1 infected CEM cells. The remaining six animals were inoculated with 1 X 10^6 uninfected cells. The experiment was terminated after 10 days. Figures 3 and 4 show the mean daily weights and tumor volumes of each group of

Figure 1. Comparison of tumor volumes (vol = $\pi/6$ LW^2) of CEM heterotransplants in irradiated and non-irradiated "nude" mice. Populations (n = 6) of mice were noculated with $0.5x10^7$ CEM cells. One group of each inoculum received 600 R ^{137}Cesium irradiation one day prior to inoculation.

FIGURE 1

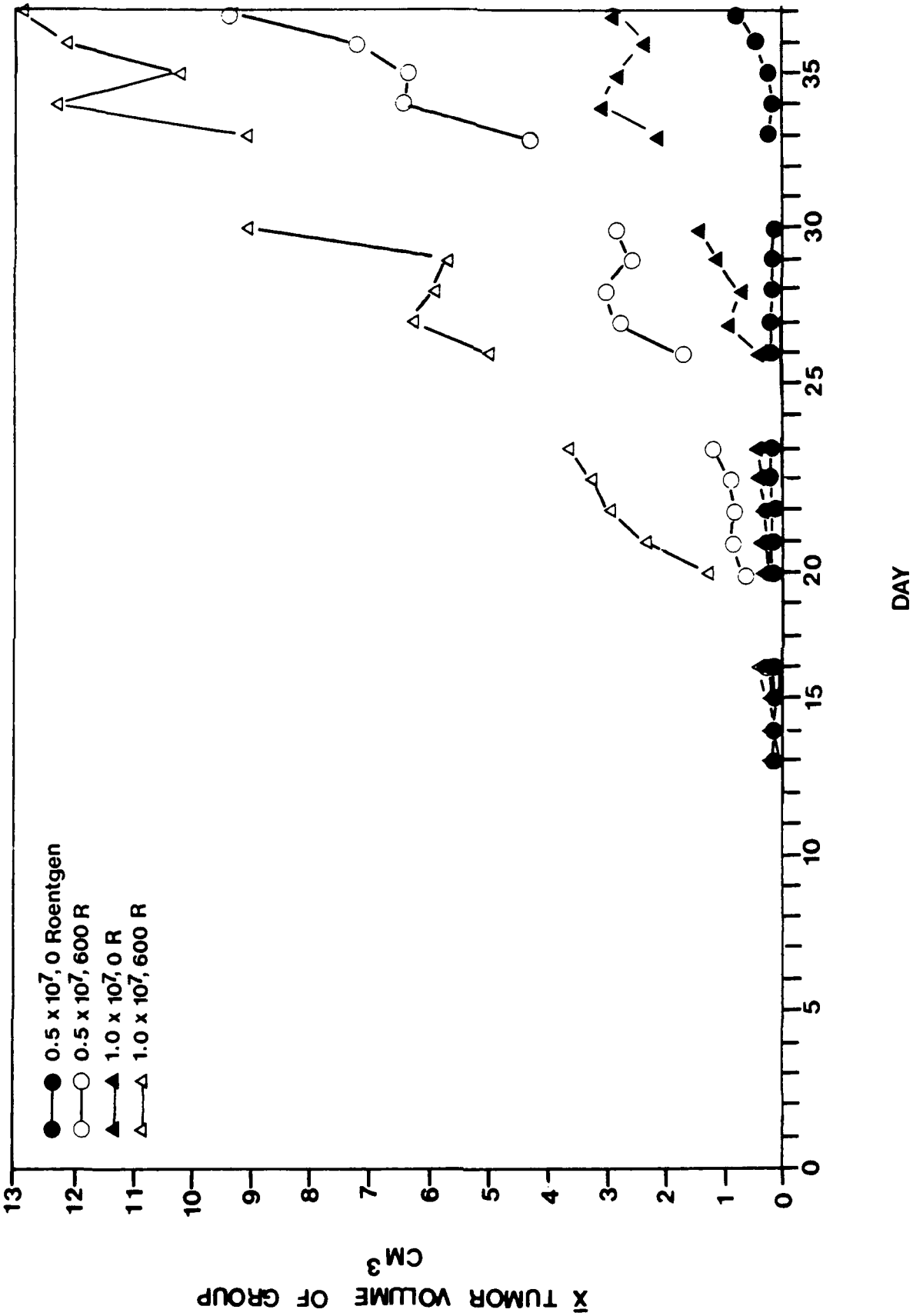

0.5 x 10^7, 0 Roentgen
0.5 x 10^7, 600 R
1.0 x 10^7, 0 R
1.0 x 10^7, 600 R
0
1
2
3
4
5
6
7
8
9
10
11
12
13
0
5
10
15
20
25
30
35
DAY
X̄ TUMOR VOLUME OF GROUP
CM3

Figure 2.Comparison of weight gain in irradiated and non-irradiated "nude" mice heterotransplanted with CEM cells. One group of each inoculum received 600 ^{137}Cesium irridation one day prior to innoculation.

FIGURE 2

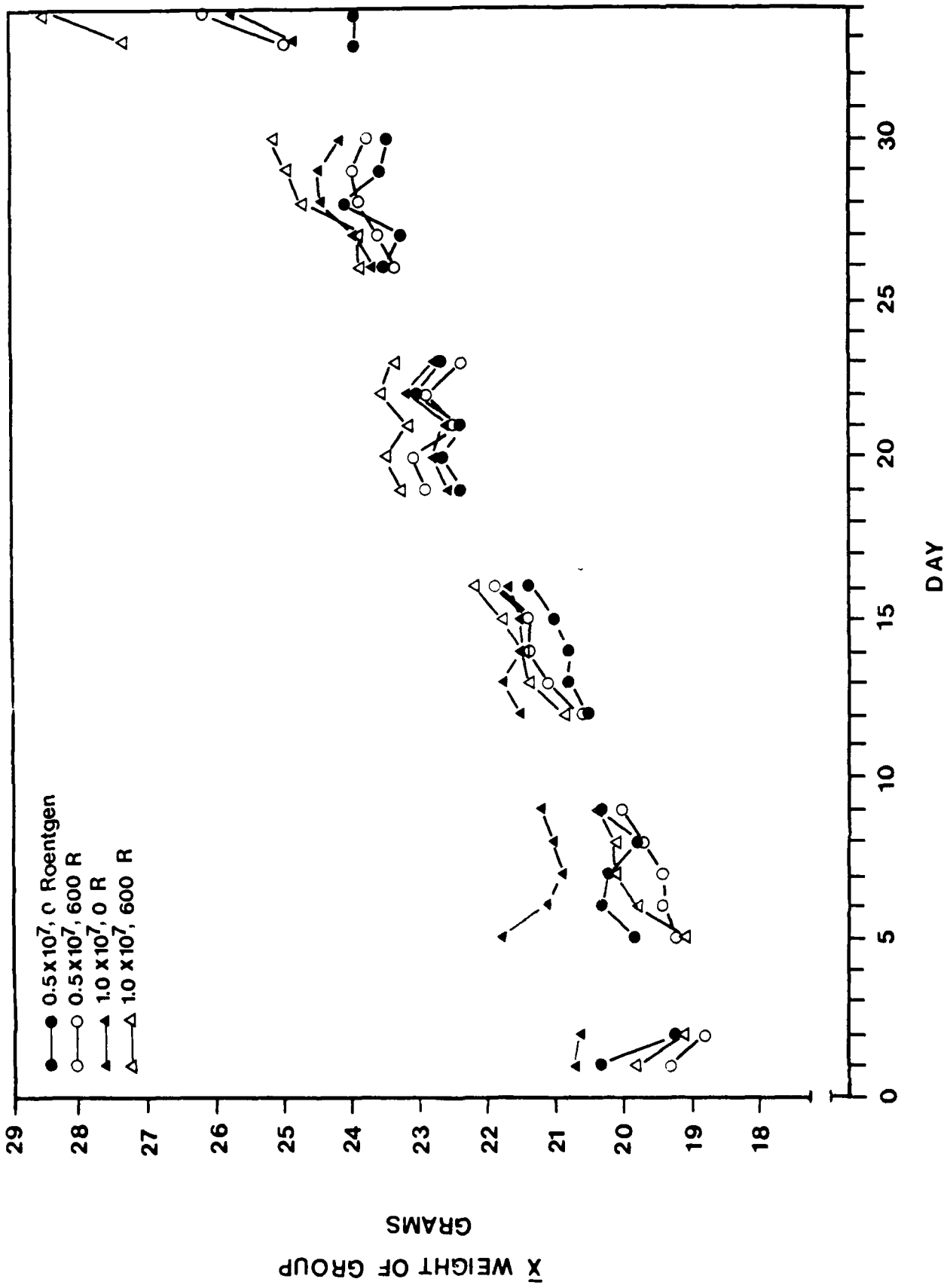

$\bar{X}$ WEIGHT OF GROUP
GRAMS
DAY
0.5 x 10^7, 0 Roentgen
0.5 x 10^7, 600 R
1.0 x 10^7, 0 R
1.0 x 10^7, 600 R
29
28
27
26
25
24
23
22
21
20
19
18
0
5
10
15
20
25
30

Figure 3. Effect of HIV-1 infection on ability of nude mice to thrive with CEM heterotransplants.

FIGURE 3

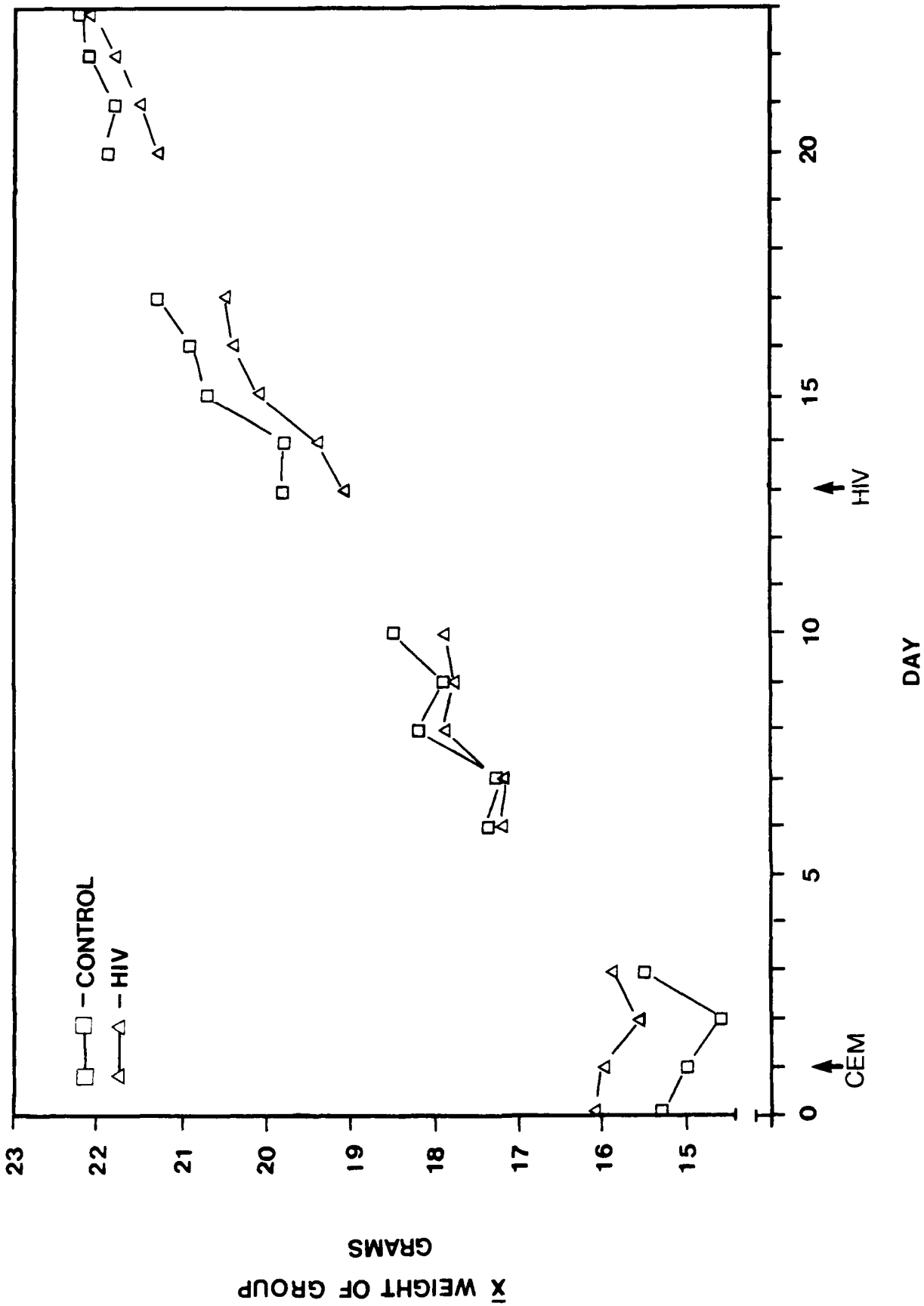
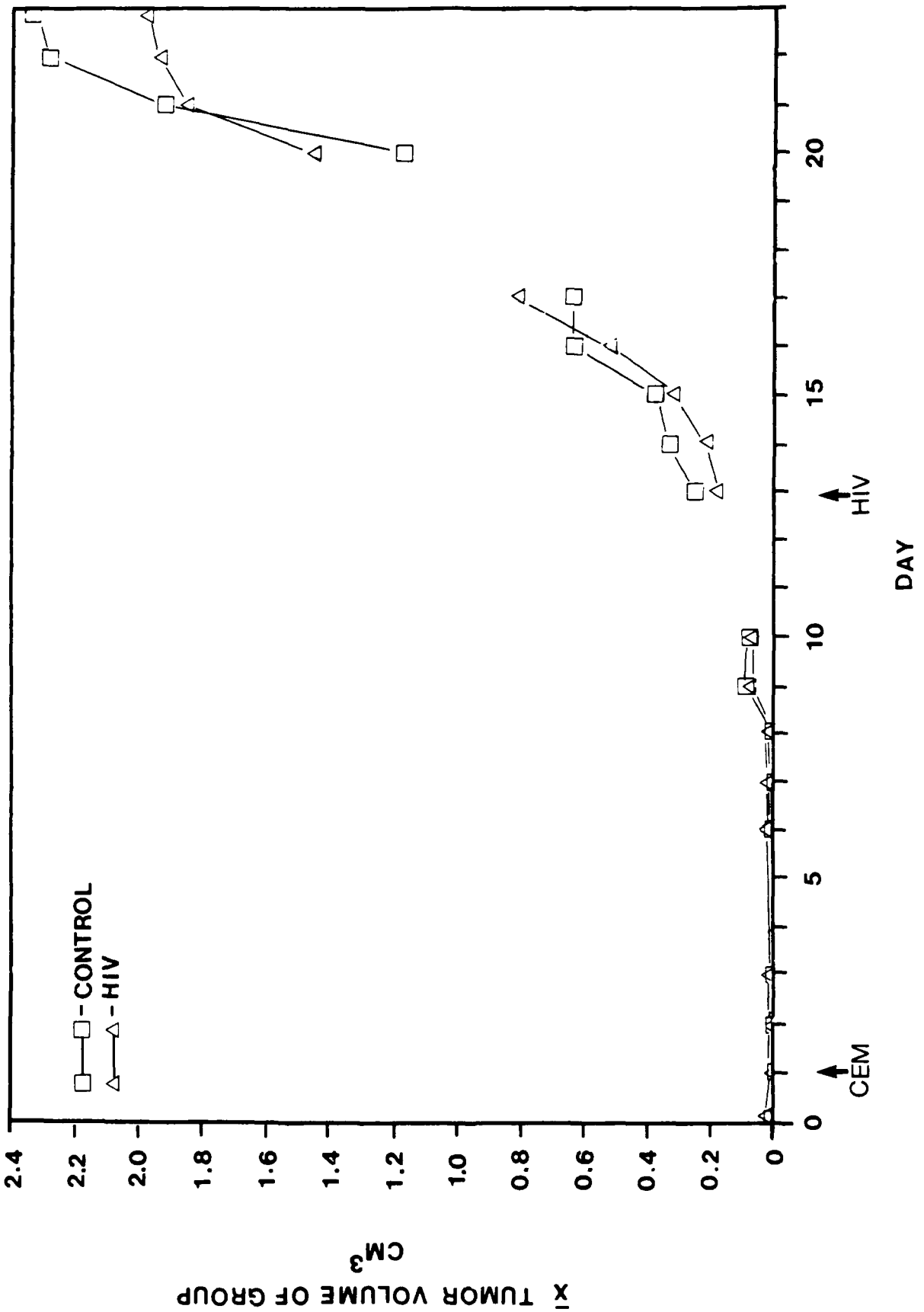

X WEIGHT OF GROUP
GRAMS
23
22
21
20
19
18
17
16
15
0
5
10
15
20
DAY
CEM
HIV
CONTROL
HIV

Figure 4. Effect of HIV-1 on tumor growth in "nude" mice heterotransplanted with CEM cells.

FIGURE 4


$\bar{x}$ TUMOR VOLUME OF GROUP
CM^3
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
DAY
CEM
HIV
- CONTROL
- HIV

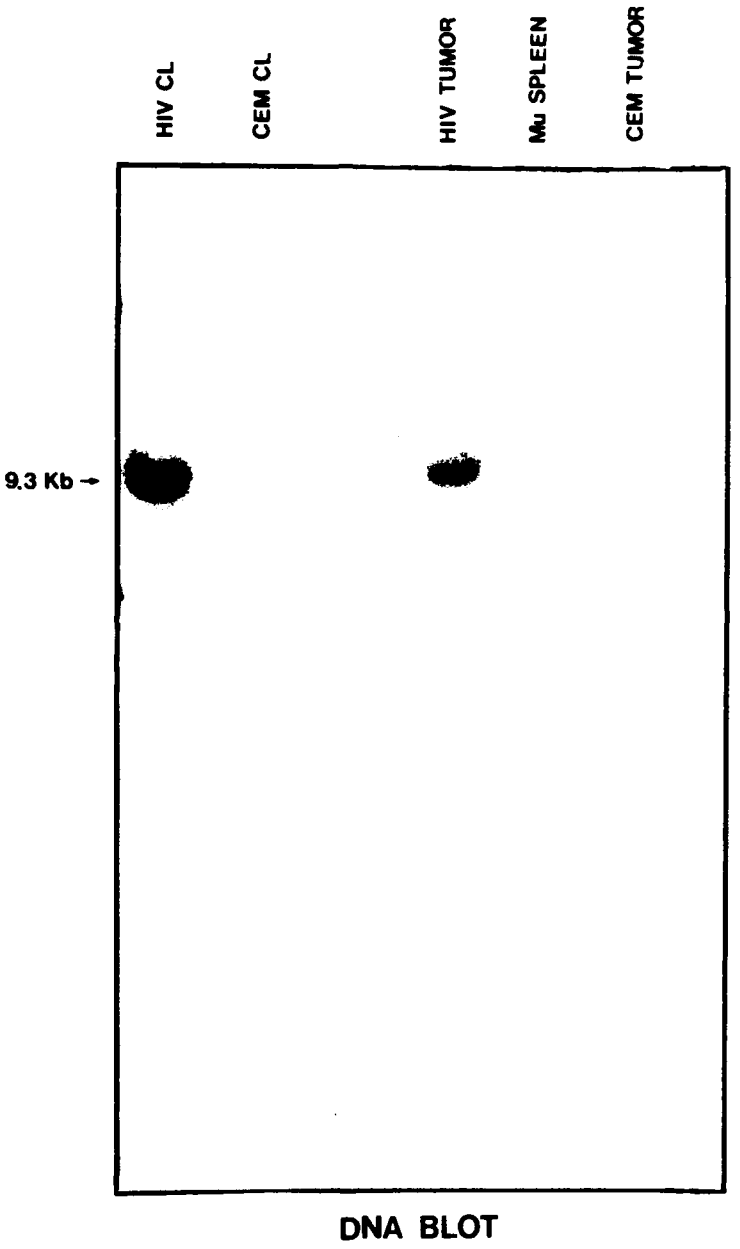
animals. No statistical differences were found in weight gain or tumor volume between any groups. Circulating p24 antigen was found in the plasma of 9 of 18 HIV-1 challenged animals with no clearly identifiable inoculum trend. The remaining six control animals were all negative. Figures 5 and 6 show the DNA and RNA blots, respectively, from a similarly HIV-1 challenged tumor after 10 days. The HIV-1 9.3 Kb proviral genome is present, as well as the 9.3 Kb genomic, 4.3 Kb envelope, and the 2.0 Kb tat and nef messenger RNA's. The results demonstrate that the virus is integrated and replicates within the CEM transplant.

C. Heterotransplantation of HIV-1 Infected CEM Cells. Thirty-six mice in three populations (n = 12) were inoculated with $1X10^7$ CEM cells which were either 100%, 50%, or 10% HIV-1 infected (derived by mixing non-infected cells with HIV-infected cells from a culture producing 10^6 infectious particles/ml). Figure 7 indicates that ,based upon tumor progression, the mice (36 infected and 6 non-infected controls) did not receive the standard amount of gamma radiation and it was subsequently determined that the equipment malfunctioned. The experiment was terminated 35 days after HIV challenge, animals exsanguinated, and plasma tested for p24 antigen. Four of the 36 infected mice produced detectable plasma antigen levels. Although the overall experiment was not interpretable, it did provide evidence for HIV-1 antigenemia from transplanted cells without target cells being present initially in the mouse (in the form of a tumor), and there is indirect evidence that the virus is able to overcome the immune defenses of the mouse. (Note: the cesium irradiator has now been updated).

Since it is theoretically possible that the antigen detected by the Abbott EIA is due to the original inoculum, an experiment was initiated to determine the time of appearance of antigen and any rise or fall in antigen levels. Populations of mice (n = 6) were injected with $1X10^7$ CEM cells infected HTLV-IIIB (100% surface antigen positive, as determined by indirect immunofluorescence, and producing 6400 pg/ml

Figure 5. Southern analysis of DNA extracted from HIV-1 infected CEM tumors in 'nude' mice. Fifteen micrograms of DNA was digested overnight with Sst I restriction endonuclease at 37° and fractionated on a 1% agarose vertical gel 20cm in length. Electrophoresis was carried out for 16 hrs at 30 V. in Tris-Acetate-EDTA buffer. Passive transfer was to nylon blotting membrane in 0.4 M NaOH, followed by hybridization with ^{32}P labeled nef–proviral sequences. Blot shows 9.0 Kb genomic provirus.

FIGURE 5



DNA BLOT

Figure 6. Northern analysis of total cellular RNA extracted from HIV-1 infected CEM tumors in "nude" mice. Twenty micrograms of RNA was denatured using DMSO/glyoxal and fractionated on a 1.2 % agarose subgel in 10 mM NaPO buffer at 90 V. for 2.5 hrs. Passive transfer was to nylon blotting membrane in 5 mM NaOH. Blot shows 9.3 Kb genomic, 4.3 Kb envelope, and 2.0 Kb tat and nef mRNA.

FIGURE 6

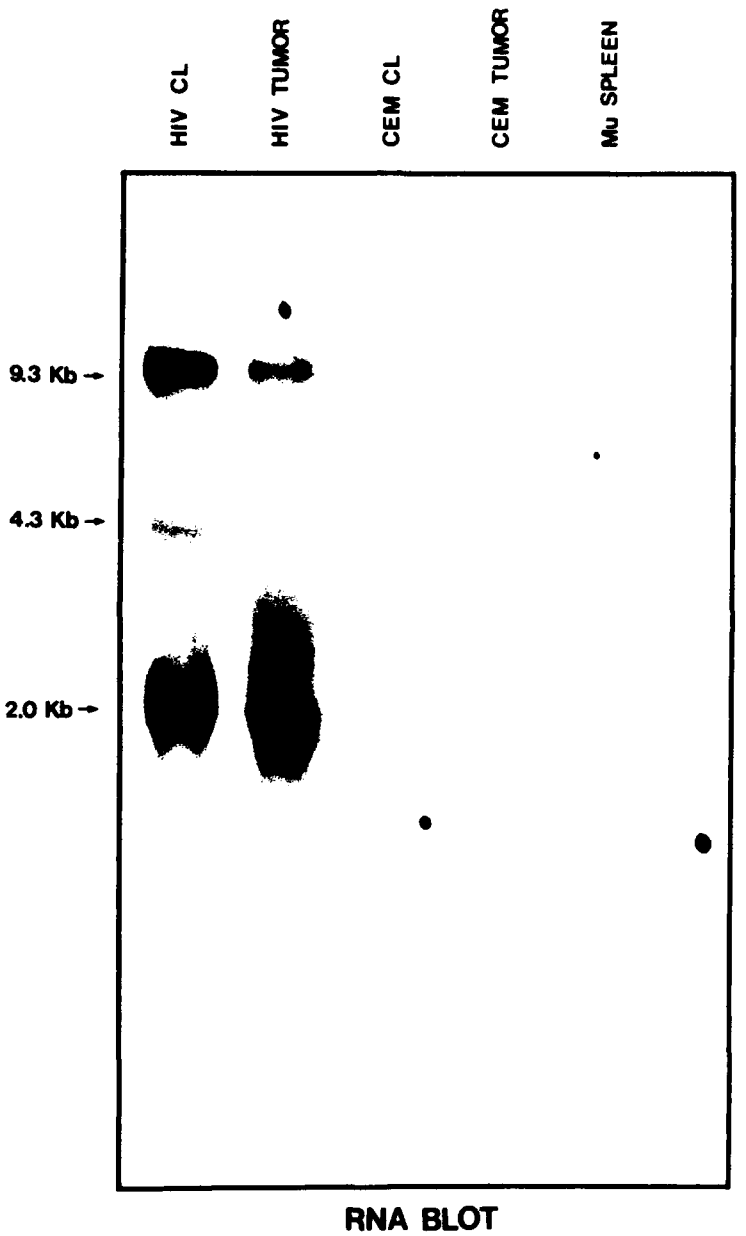
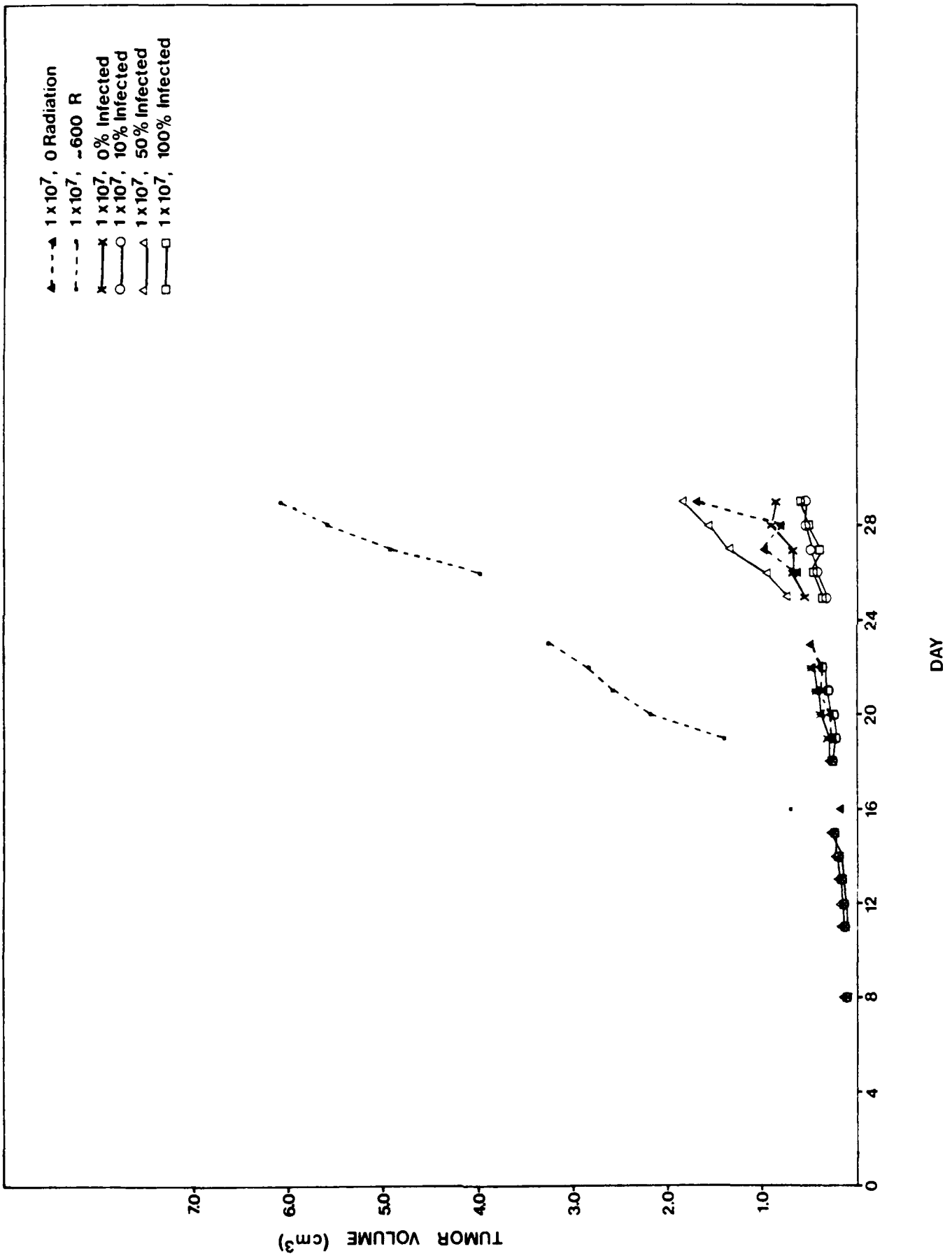

HIV CL
HIV TUMOR
CEM CL
CEM TUMOR
Mu SPLEEN
9.3 Kb
4.3 Kb
2.0 Kb
RNA BLOT

Figure 7. Mean tumor volumes of CEM heterotransplants with varying percentages of HIV-infected cells. Superimposed on graph are tumor volumes from previous experiments showing reduced tumor growth in non-irradiated mice.

FIGURE 7


1 x 10^7, 0 Radiation
1 x 10^7, ~600 R
1 x 10^7, 0% Infected
1 x 10^7, 10% Infected
1 x 10^7, 50% Infected
1 x 10^7, 100% Infected
TUMOR VOLUME (cm^3)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
20
24
28
DAY

p24 antigen *in vitro*). Control populations (n = 3) were injected with $1X10^7$ uninfected CEM cells. At 24, 48, and 72 hours and 7,14, and 21 days post transplantation one infected and one control population was sacrificed and plasma collected. No antigen was detected in any animal, so although we can conclude that antigen detected previously was not due to inoculum, we could not answer any of the other questions for which the experiment was designed. Retrospectively, we know from later experiments that the lack of antigenemia was most likely the result of using only infected cells in the inoculum without providing uninfected target cells, and possibly the result of using a moderately virus-producing culture as compared to cultures used in later experiments.

D. Kinetics of HIV-1 Infected Cells in Nude Mice. Since direct evidence existed from past experiments that HIV-1 will replicate *in vivo* and that 100% infected culture may not produce the most consistent antigenemic state, we inoculated three populations of mice (n = 6) with $1X10^7$ CEM cells (10%, 50%, and 100%) HIV-1 infected cells derived from a culture producing approximately 1550 pg/ml of p24 antigen. Two additional groups consisted of an uninfected control group and a group inoculated with 1 X 10^7 uninfected CEM cells and then challenged at tumor onset (day 7) with $0.5X10^7$ 100% infected cells producing 976 pg/ml p24 antigen *in vitro*. The animals were followed for 23 days. Transient differences were seen in tumor volumes (Figure 8) and marginally significant differences in weights were seen between the 100% infected inoculum group and the control group (Figure 9). Plasma was assayed for p24 antigen and the results are shown in Table IV.

All populations infected with HIV-1 produced antigenemic mice. The average antigen level (pg/ml) of each group varied inversely with the percent of infected cells in the inoculum. In those mice where sufficient plasma remained for a neutralization assay all reactive plasma reverted to non-reactive. This provides strong evidence that antigenemia in the mice is due to HIV-1 and not the result of

Figure 8: Mean tumor volumes of CEM heterotransplants with varying percentages of HIV-infected cells. All populations were inoculated with 1 X 10^7 cells. Population E was challenged at tumor onset with 0.5 X 10^7 100% infected cells.

A = Control, 0% infected

B = 10% infected

C = 50% infected

D = 100% infected

E = 0% initially, followed by 100% challenge

FIGURE 8

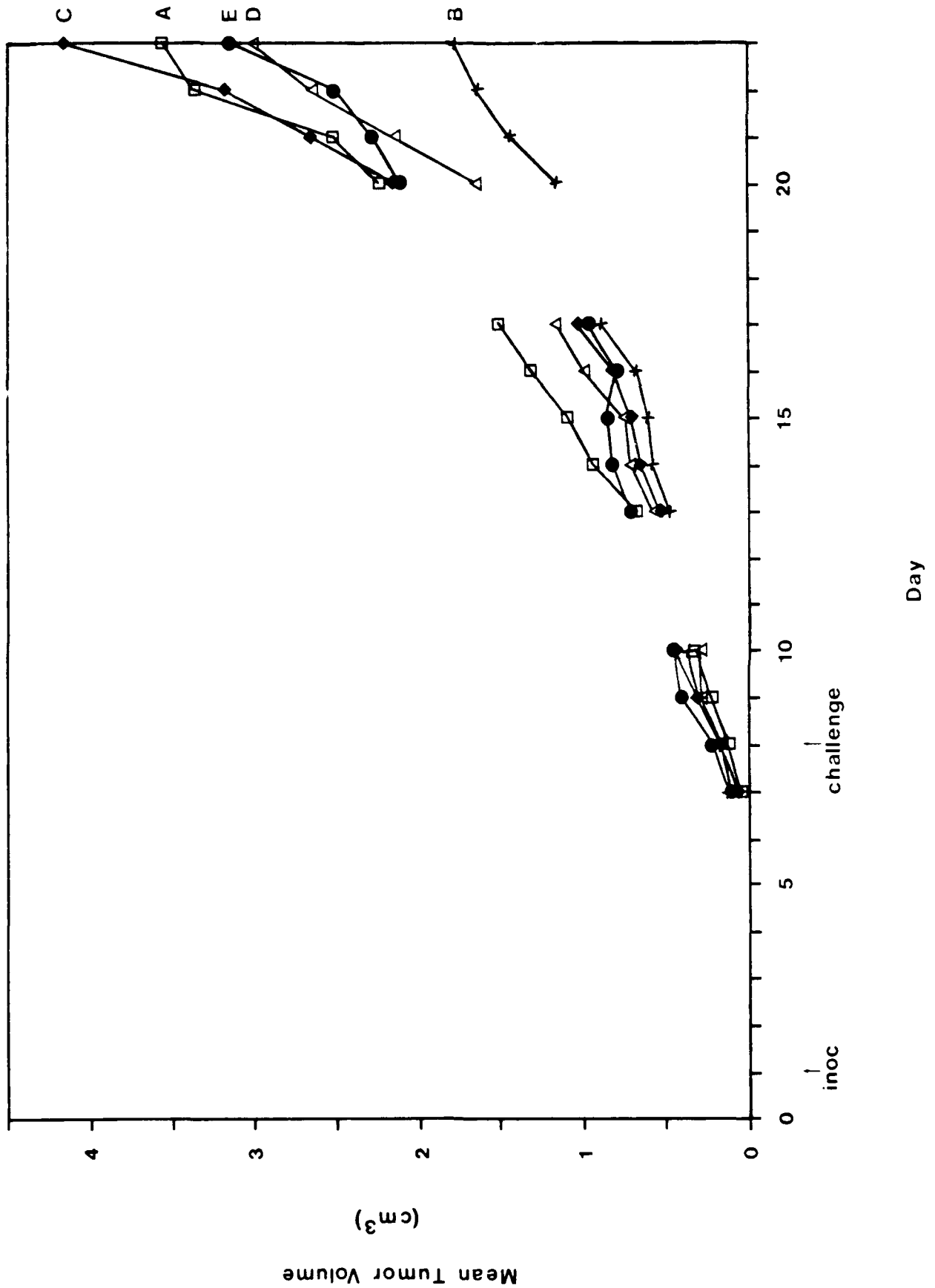

C
A
E
D
B
0
1
2
3
4
Mean Tumor Volume
(cm3)
0
5
10
15
20
Day
inoc
challenge

Figure 9: Weight gain in "nude" mice heterotransplanted with varying percentages of HIF-infected CEM cells. All populations were inoculated with 1 X 10^7 cells. Population E was challenged at tumor onset with 0.5 X 10^7 100% infected cells.

A = Control, 0% infected

B = 10% infected

C = 50% infected

D = 100 % infected

E = 0% initially, followed by 100% challenge

FIGURE 9

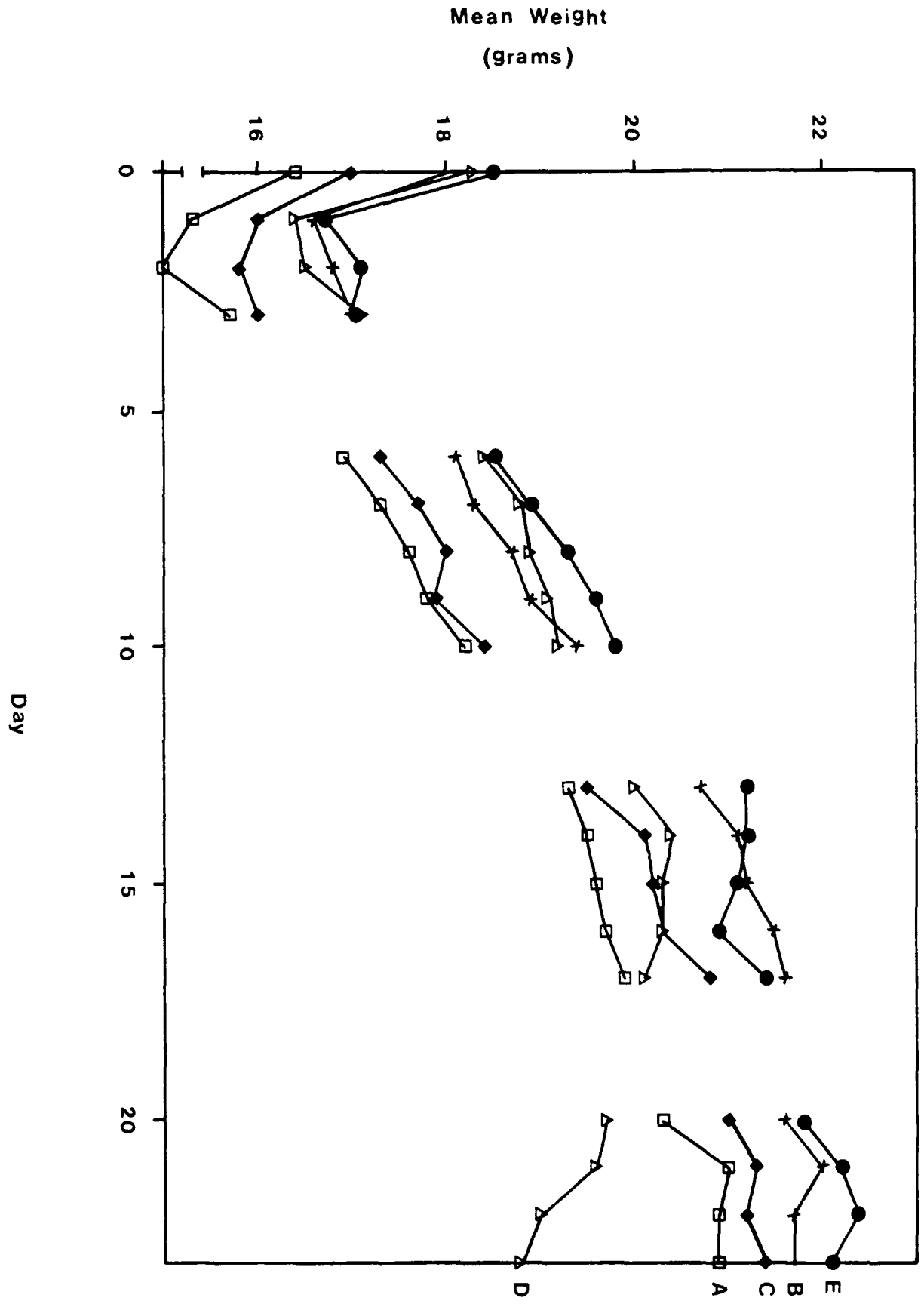


TABLE IV

CEM Inoculum	% Inoculum with HIV III_B	Total Mouse Pop	% p24 Ag positive	Plasma A_g pg/ml (S.E.M.)	Ag After Ab neutralization
1×10^7	0	5	0	-	not detected
1×10^7	10	6	100	129.6 (8%)	not detected
1×10^7	50	6	83	70.4 (21%)	not detected
1×10^7	100	6	50	15.4 (8%)	not detected
1×10^7	0.5×10^7*	6	17	9	not detected

S.E.M. = Standard Error of Mean
*Challenge after palpable tumor detected

activation of a latent murine virus. Samples of tissues were reacted with sheep anti-gp120 and anti-p24 as well as rabbit anti-p24 (supplied by Dr. J. Stewart, Abbott Laboratories). Figure 10 demonstrates gp120 staining of an HIV-infected CEM tumor derived from a mouse with a plasma antigen titer of 147 pg/ml. Figure 11 exhibits strong gp120 staining of apparent mouse macrophages. Similar immunohistochemical staining is seen with both sheep and rabbit p24 antibodies. Transmission electron microscopy confirmation of HIV-1 is seen in Figure 12. The bar-shaped nucleoid of a lentivirus is clearly present. Virus was only visualized in this apparently intracellular vacuole, none were seen on CEM lymphoma cell membranes. The only histopathology seen was in the antigenically stimulated spleens of infected mice. Figure 13 demonstrates a reactive follicular center surrounded by a hyperplastic zone suggestive of a fairly strong B-cell response. No mouse antibodies to HIV have been studied to date.

To establish that the p24 assay was detecting replicating virus and not merely detecting residual protein from the initial inoculum, four ^{137} Cs-irradiated mice were injected intraperitoneally with 0.5 ml each of cell-free tissue culture supernatant from a CEM/HIV-1 culture producing 500,000 pg/ml p24 antigen. One mouse was injected ip with uninfected CEM cell culture supernatant. Challenged mice were sacrificed at 24 hrs., 48 hrs., 7 days, and 14 days. Plasma from the control mouse was collected at 48 hrs. and was non-reactive for p24 antigen. In the challenged mice, only at 24 hrs. was antigen detectable (2.9 pg/ml); all other plasma

Figure 10. Stained plastic section of HIV-1 infected CEM tumor transplanted into a "nude" mouse reacted with sheep polyclonal antibody to gp120 HIV envelope protein. Peroxidase conjugated secondary antibody was developed with diaminobenzidine as substrate.

FIGURE 10

Figure 11: Stained plastic section of spleen from "nude" mouse heterotransplanted with HIV-infected CEM cells reacted with sheep polyclonal antibody to gp120 HIV envelope protein. Peroxidase conjugated secondary antibody was developed with diaminobenzidine as substrate. Positive cells appear to be plasma cells.

FIGURE 11

Figure 12. Transmission electron micrograph of HIV-1 infected CEM tumor transplanted into a "nude" mouse. Virus particles are visible in intracellular vacuole. Arrow indicates typical bar-shaped nucleoid of a lentivirus.

Magnification = 31,900 X

FIGURE 12

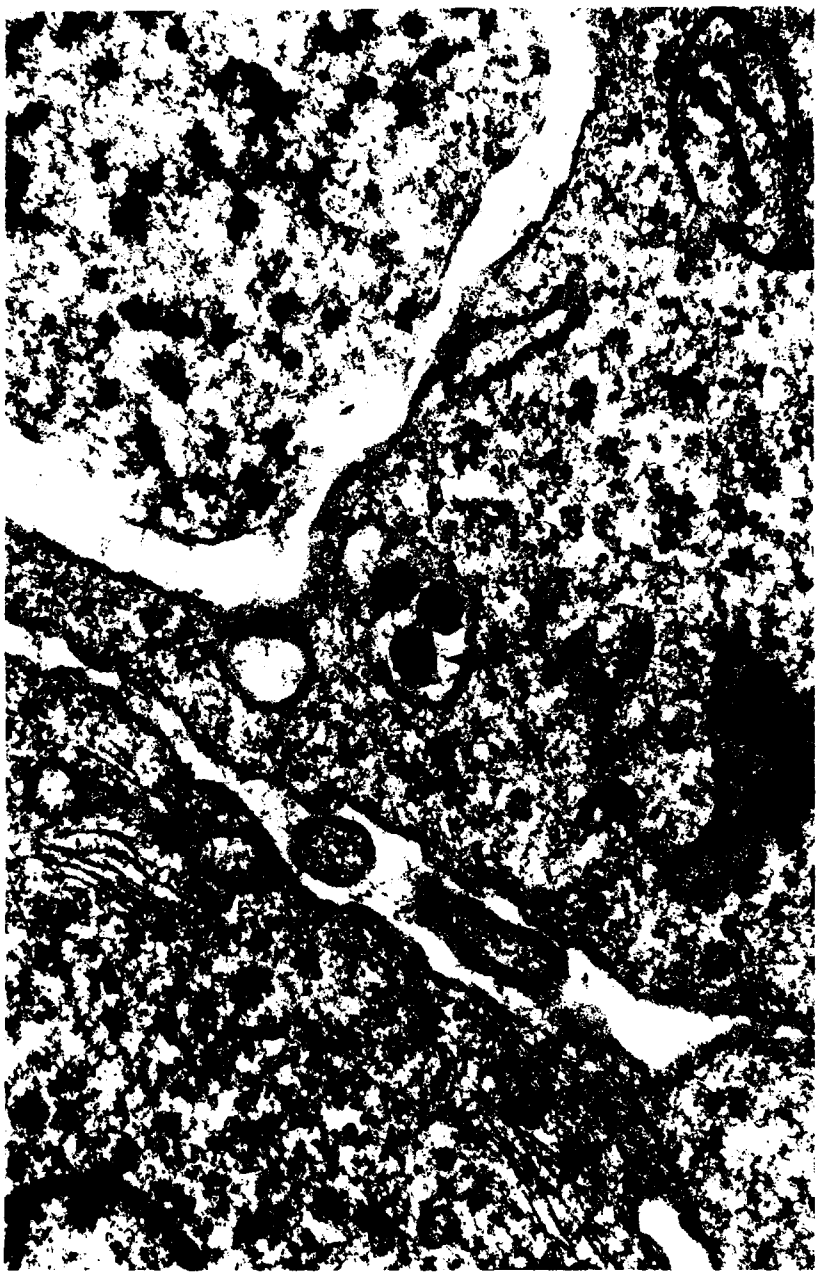


Figure 13. Paraffin hematoxylin-eosin stained section of spleen from a 'nude' mouse transplanted with HIV-infected CEM cells. Animal was plasma p24 antigen positive. Section shows reactive follicular center surrounded by hyperplastic zone.

FIGURE 13

was below detectable limits. To define a p24 antigenemia curve of viral persistence *in vivo*, 45 irradiated nude mice were transplanted with $1X10^7$ CEM cells, 90% uninfected and 10% of which were from a culture producing 193,000 pg/ml p24 antigen *in vitro*. Six control mice were inoculated with $1X10^7$ uninfected CEM cells. One population (n = 6) was sacrificed by exsanguination at days 3, 5, 7, 14, 21, 28, 42, and 63 post inoculation. Plasma was assayed for p24 antigen. Figure 14 shows the curve obtained and its relationship to tumor volume.

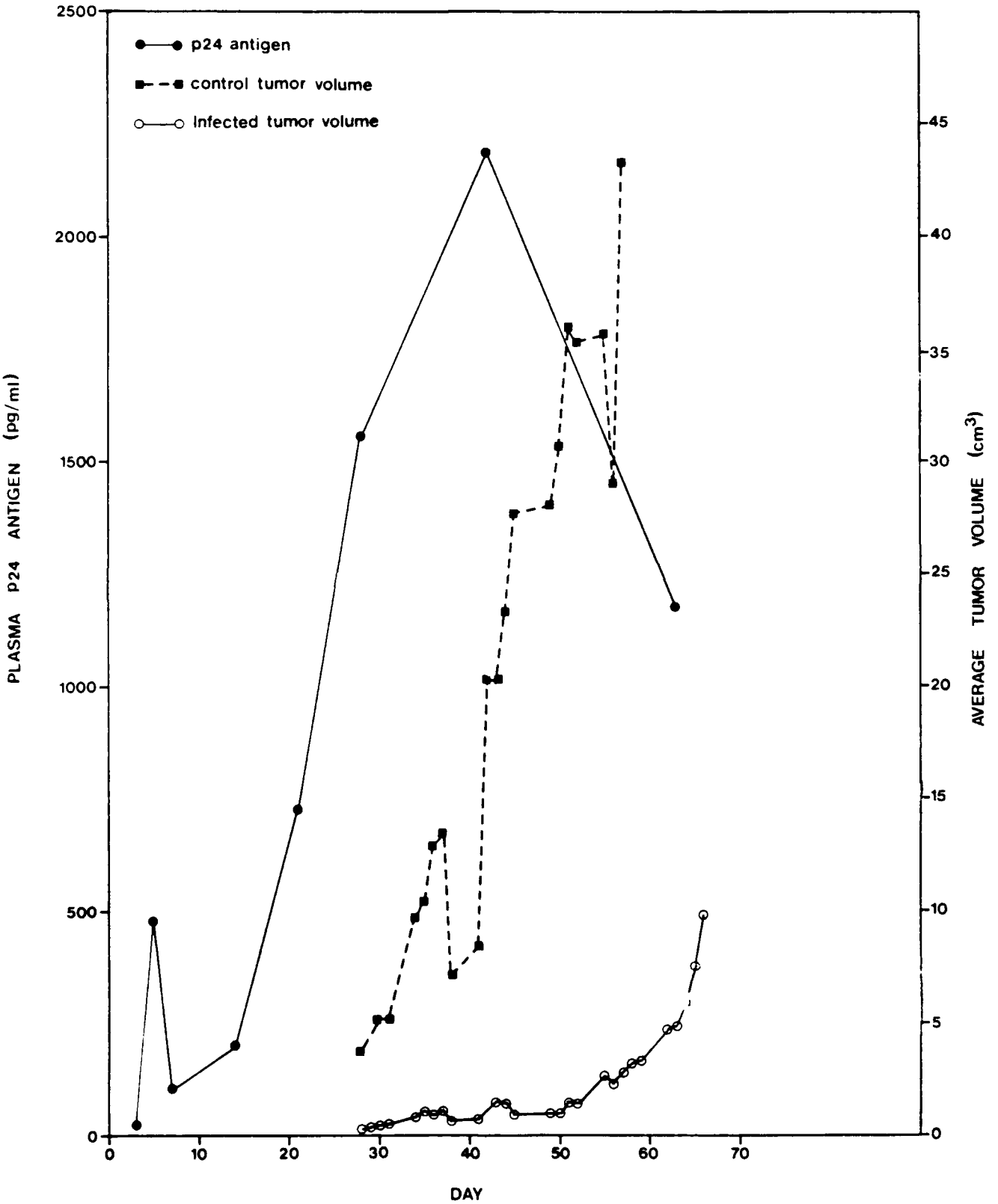
E. Adaptation of BALB/c Mice to HIV-1 Susceptible Cells. The intent of this work was to develop a system in normal mice to grow HIV-1. In collaboration with Dr. Barney Graham, Dept. of Medicine, VUMC, the approach to adapt normal BALB/c mice to HIV-1 susceptible cell lines was instituted.

Initial efforts involved exposing neonatal mice to human CD4 + CEM cells. A total of 36 mice from 9 litters were given 2×10^7 CEM cells intravenously via the facial vein within 12 hours after birth. They were then challenged with 2×10^7 CEM subcutaneously immediately to 4 weeks of age. This result was unsuccessful as was a similar approach using 17 mice from 3 litters of CEM immune dams.

Subsequent work has focused on immunomodulation of adult mice. The preparation of mice (BALB/c female retired breeders) includes sublethal irradiation (450 TBR) and depletion of the murine CD4 + lymphocyte population with an L3T4 specific monoclonal antibody, GK 1.5. The hybridoma secretes a rat IgG_{2b} antibody which we have produced in ascites using nude mice and irradiated BALB/c mice. Over the last 6 months, we have produced over 40 ml of ascites containing 1.0 g/dl of immunoglobulin. Mice are immunodepleted using 100 mcg of GK 1.5 intraperitoneally on 3 successive days then maintained with weekly injections of 250 mcg. To date, 13/13 mice undergoing this regimen have developed subcutaneous tumors after inoculation with 2×10^7 CEM and 4/4 with the human CD4 + monocyte line U937.

Figure 14. Plasma p24 antigen levels in HIV-infected "nude" mice showing effect of antigenemia on CEM tumor growth.

FIGURE 14


p24 antigen
control tumor volume
Infected tumor volume
PLASMA P24 ANTIGEN (pg/ml)
AVERAGE TUMOR VOLUME (cm3)
DAY
2500
2000
1500
1000
500
0
45
40
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70

Mice treated with GK 1.5 or 450 TBR alone will not develop tumors. Groups of 4 mice each were treated in this manner, then after 6 weeks underwent the full immunodepletion regimen with GK 1.5 and TBR. Still no tumors evolved, implying that once immune memory cells have been established, depletion of the CD4+ subset is insufficient to induce tolerance. Mice that were not maintained with additional doses of GK 1.5 began rejecting the tumor during week 3 after inoculation. The tumors in mice maintained with injections of GK 1.5 continued to grow exponentially beyond week 5.

Mice with CEM or U937 tumors were prepared to assess susceptibility to HIV-1 infection. Of the 4 mice in each grup, 3 were inoculated with 8×10^6 CEM persistently infected with the HTLV-IIIB strain by direct tumor injection. One mouse in each group was injected with uninfected CEM cells. The mice were assessed by measuring p24 antigen in serum. By day 16, all mice had positive assays for antigen. The CEM bearing mice had higher titer than U937 based on O.D. in the Abbot kit. Interestingly, the uninfected mouse in the CEM cage had a positive antigen assay at day 16, suggesting transfer from one of the infected cagemates. The U937 control mouse died during a day 6 venipuncture and so a similar event did not have the opportunity to occur in that cage. Immunocytochemistry, histology, and other studies are still pending.

III. COMPARISON TO GOALS

In the original contract technical proposal, the following specific aims for the first year were detailed as follows:

1. To establish the optimum conditions for HIV permissive cells to grow in athymic 'nude' rodents.
2. To establish optimum conditions for HIV to replicate in heterotransplants.

3. To refine and apply established techniques (laboratory tests) to assess the replicative cycle of HIV.
4. To determine the pathological changes of a broad spectrum of tissues, but especially the brain and spinal column in test animals as compared to controls.
5. To determine the overall mortality in test animals as compared to controls.
6. To determine the potential for anti-HIV therapy.
7. To evaluate heterotransplants for use in HIV isolation from patients.

(two additional specific aims which involved neurological studies were deleted in later negotiations.) Based on our results to date, especially in our use of a commercially available and clinically useful laboratory test (Abbott Diagnostics, p24 antigen assay) to study HIV-1 replication *in vivo*, we believe that our goals have been achieved with the exception of those involving patient material. We now feel that our *in vivo* system is ready for those evaluations, and initial studies are underway. (see Experimental Methods section).

CONCLUSIONS

I. IMPLICATIONS

During the first half of this contract, we have demonstrated that by using a simple approach we can maintain HIV-1 replication in mice for at least two months. This murine model of HIV-1 infection can be used to study most, if not all, of the parameters listed in Table 1. The CEM xenotransplanted nude mouse system is based upon sound and established methods. The results displayed in Figure 14 indicate that high titer virus has a direct effect on the growth of CEM cells *in vivo* since tumor progression is inhibited while antigenemia is increasing. This finding is important for two reasons: firstly, while the circulating virus load is high, therapy can be used to interfere with virus replication and decrease the p24 antigen levels, and secondly, since the virus appears to be effected by the mouse immune system

(cellular, humoral or both), identification of factors involved is prudent as this defense may have implications for human disease.

II. FUTURE WORK

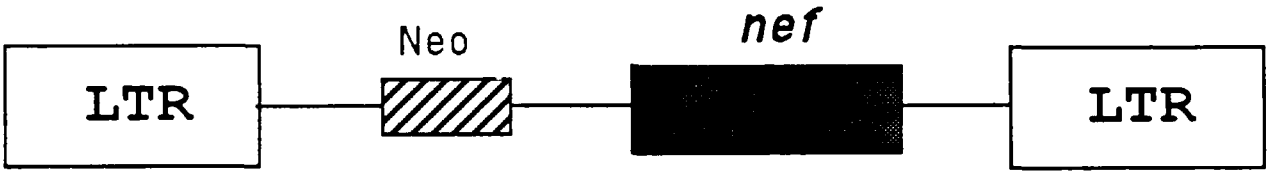
It is clear that the simple use of commercially available kit to measure a clinically relevant parameter of HIV-1 infection provides a means to assay antiviral agents. The strategy for implementation will soon be in place, and progress through the remainder of this contract. Based on the results displayed in Table IV and Figure 14, cultures of HIV-1 infected CEM cells will be adjusted and maintained to produce virus at levels where the minimum amount of virus is used to produce a 100% infected mouse population. Since the antivirals are limited in quantity, initial experiments will consist of short-term screens using compounds provided by collaborators Dr. Raymond Schinazi (Atlanta Veterans Administration Medical Center) and Dr. Michael Chirigos (USAMRIID, Fort Detrick). The use of innovative molecular means of antiviral therapy will also be investigated. Dr. Jeff Holt of our institution (Depts. of Cell Biology and Pathology) will be instrumental in assisting in SIN Vector targeting and antisense oligonucleotide means of inhibiting the expression of viral genes *in vivo* (Fig. 15). The first two oligos (phosphorothioate synthesized) are currently being prepared and one of these sequences has previously been shown to be active against HIV *in vitro* (69).

Additional investigations will involve studies on the mouse immune system. These studies will involve both humoral and cellular aspects. Preliminary data from these studies will be the focus of this next USAMRDC contract submission. Studies involving patient material will continue. Emphasis will be put on stimulation of lymphocytes to produce antigen in antibody positive but plasma antigen negative patients. Summary of work to date is in the appendix.

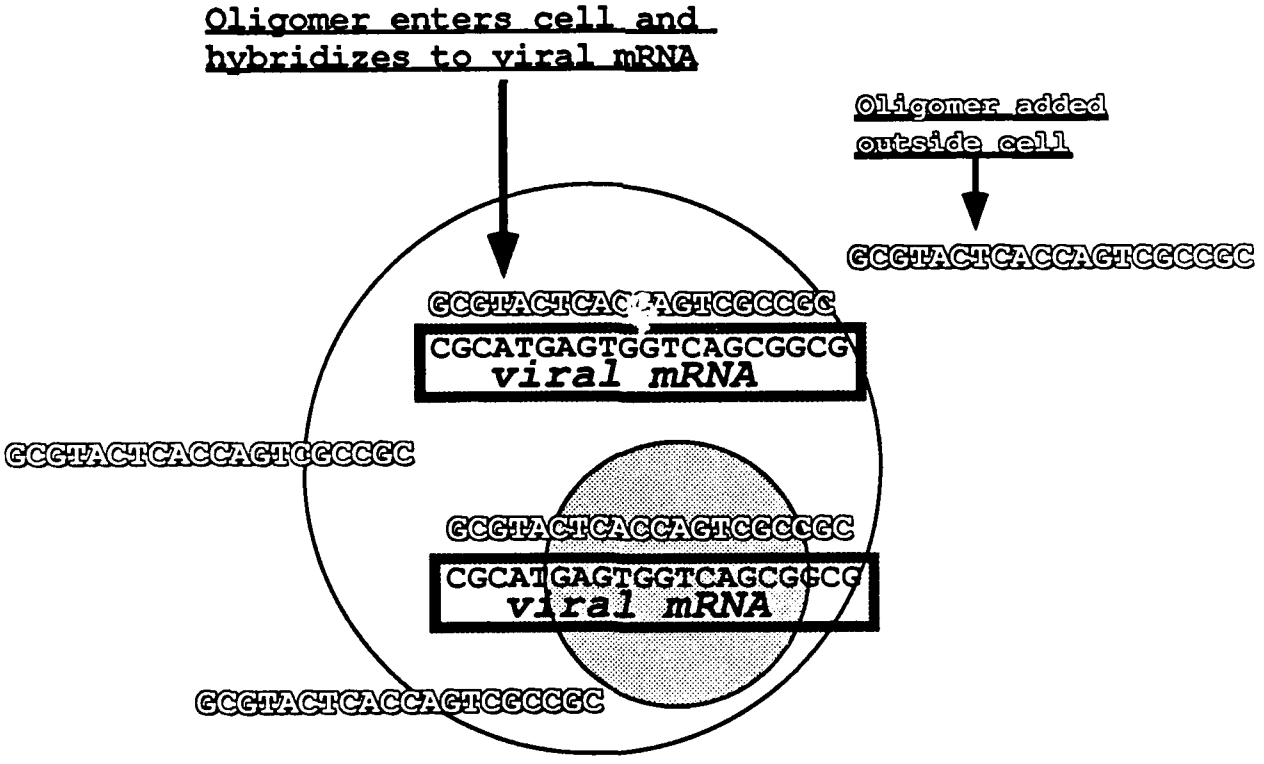
Figure 15. A. Suicide inactivation vector containing the nef region of the HIV-1 genome.

B. General mechanism for the inhibition of gene expression by exogenous antisense oligomers. Both sense and antisense of gene sequence shown is currently under synthesis.

FIGURE 15


LTR
Neo
nef
LTR

A


Oligomer enters cell and hybridizes to viral mRNA
Oligomer added outside cell
GCGTACTCACCAGTCGCCGC
GCGTACTCACCAGTCGCCGC
CGCATGAGTGGTCAGCGGCG
viral mRNA
GCGTACTCACCAGTCGCCGC
GCGTACTCACCAGTCGCCGC
CGCATGAGTGGTCAGCGGCG
viral mRNA
GCGTACTCACCAGTCGCCGC

B

III. IMPACT ON PROBLEM TOPIC

During the first year of this contract, we have been successful in defining baselines for the replication of HIV-1 in heterotransplanted CEM cells. Although the exact infectious dose of HIV to be given to obtain optimum antigen levels for antiviral testing remains to be worked out and the role of the murine immune system remains to be explored, we believe our model with its easily defined antigenemia curve provides the best method for initial studies of antivirals.

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APPENDIX

1. Summary of p24 Antigenemic Mice
2. Table of Plasma p24 Antigen Levels of Patients Involved in Study
3. Letter of Collaboration - Dr. R. Schinazi
4. Letter of Collaboration - Dr. M. Chirigos
5. Abstract from 28th ICAAC
6. Abstracts from V Int'l AIDS Conference

SUMMARY OF p24 ANTIGENEMIC MICE

MOUSE (EXP POP. #)	#CEM CELLS INOC	#CEM/HIV-III_B CHALLENGE INOC	DAY OF TUMOR ONSET	TUMOR VOL AT SACRIFICE	#DAYS VIRUS PRESENT IN MOUSE	ABS λ 492/600 (ABBOTT ASSAY)	ABS AFTER NEUTRAL ASSAY	p24 Antigen pg/ml
IIID4	1×10^7	1×10^6	9	.11	10	>2.000	nd*	>200
IVB0	$.5 \times 10^7$	1×10^4	7	.68	10	1.244	nd	174.6
IVC1	$.5 \times 10^7$	1×10^5	8	.51	10	.220	nd	25
IVC2	$.5 \times 10^7$	1×10^5	7	1.60	10	1.071	nd	149.6
IVC3	$.5 \times 10^7$	1×10^5	7	.73	10	.153	nd	15
IVC4	$.5 \times 10^7$	1×10^5	8	.47	10	>2.000	nd	>200
IVD0	$.5 \times 10^7$	1×10^6	8	1.70	10	.975	nd	136
IVD2	$.5 \times 10^7$	1×10^6	8	.95	10	.164	nd	17
IVD4	$.5 \times 10^7$	1×10^6	8	1.70	10	.148	nd	14.6
IVD5	$.5 \times 10^7$	1×10^6	8	2.50	10	.810	nd	111.6
		% INFECTED CELLS						
VC0	1×10^7	50	12	3.00	39	.508	nd	
VC2	1×10^7	50	10	6.70	39	.245	nd	
VIB1	1×10^7	50	7	7.30	35	.166	.049	28.6
VIB4	1×10^7	50	8	10.30	35	.201	.053	22.6
VIC3	1×10^7	10	6	1.00	35	.178	nd	19
VIE3	1×10^7	100	6	1.80	35	.263	.051	31.6

*not determined

TABLE OF PLASMA p24 ANTIGEN LEVELS OF PATIENTS INVOLVED IN STUDY

MOUSE (EXP. POP. #)	#CEM CELLS INOC	% INFECT-ED CELLS	DAY OF TUMOR ONSET	TUMOR VOL AT SACRIFICE	#DAYS VIRUS PRESENT IN MOUSE	ABS 492: 600 (ABBOTT ASSAY)	ABS AFTER NEUTRAL ASSAY	p24 ANTIGEN pg/ml
VIII BO	1×10^7	10	6	2.20	22	1.493	.051	213
VIII B1	1×10^7	10	6	0.47	22	1.048		147
VIII B2	1×10^7	10	6	1.30	22	.273	.053	27.6
VIII B3	1×10^7	10	6	1.40	22	.510	.047	65
VIII B4	1×10^7	10	6	3.20	22	1.243	.047	176.6
VIII B5	1×10^7	10	6	2.10	22	1.053	.055	148
VIII C0	1×10^7	50	7	6.50	22	.607	.047	80
VIII C1	1×10^7	50	7	5.00	22	1.468	.047	209
VIII C3	1×10^7	50	7	6.80	22	.310	.057	35
VIII C4	1×10^7	50	7	1.90	22	.162	046	13
VIII C5	1×10^7	50	7	3.60	22	.175	nd	15
VIII D0	1×10^7	100	7	2.50	22	.160	nd	13
VIII D4	1×10^7	100	6	1.10	22	.198	nd	18
VIII E4	1×10^7 non-HIV	0.5×10^7 HIV CHALLENGED	6	1.10	15	.134	.055	9

Mouse (Exp,Pop,#)	#CEM Cells Inoc	%Infected Cells	Day of Tumor onset	Tumor Vol at Sacrifice	#Days Virus Present in Mouse	ABS Δ 492 600 (Abbott Assay)	ABS After Neutral Assay	p24 Antigen pg/ml
XB1	$1x10^7$	10			3	0.122	nd	7.4
XB2	$1x10^7$	10			3	0 828	0 058	96.4
XB5	$1x10^7$	10			3	0 527	nd	59
XC1	$1x10^7$	10			5	1 226	0 053	652
XC2	$1x10^7$	10			5	1 024	nd	1104
XC3	$1x10^7$	10			5	1 035	nd	312
XC4	$1x10^7$	10			5	>2 000	nd	over 1,000
XC5	$1x10^7$	10			5	1 525	0 057	818 4
XD0	$1x10^7$	10	8	0 09	7	0 271	nd	26 4
XD2	$1x10^7$	10	10	0 06	7	1 560	0 070	190
XD3	$1x10^7$	10	7	0 05	7	1 122	nd	13 5
XD4	$1x10^7$	10	8	0 04	7	0 642	nd	186
XE0	$1x10^7$	10	10	+	14	0 125	nd	8 4
XE1	$1x10^7$	10	7	+	14	0 343	nd	36
XE2	$1x10^7$	10	10	+	14	0 312	nd	31 4
XE4	$1x10^7$	10	8	0 28	14	0 991	0 053	520
XE5	$1x10^7$	10	10	0 40	14	1 022	nd	605 7
XF0	$1x10^7$	10	8	0 26	21	1 119	nd	590 4
XF1	$1x10^7$	10	7	0 17	21	1 441	0 059	1 576
XF2	$1x10^7$	10	8	0 19	21	0 864	0 053	448 8
XF3	$1 10^7$	10	7	0 26	21	1 528	nd	81 36
XF4	$1x10^7$	10	7	0 15	21	1 488	nd	180
XF5	$1x10^7$	10	7	0 11	21	1 329	nd	724

MOUSE (EXP. POP. #)	#CEM CELLS INOC	% INFECTED CELLS	DAY OF TUMOR ONSET	TUMOR VOL AT SACRIFICE	#DAYS VIRUS PRESENT IN MOUSE	ABS Δ492: 600 (ABBOTT ASSAY	ABS AFTER NEUTRAL ASSAY	p24 ANTIGEN pg/ml
XG1	1×10^7	10	8	0.47	28	0.949	nd	5392
XG2	1×10^7	10	10	0.17	28	0.938	0.064	1043,2
XG4	1×10^7	10	7	0.26	28	1.691	nd	920
XG5	1×10^7	10	7	0.33	28	1.723	0.085	1966.4
XH1	1×10^7	10	7	0.26	42	1.298	nd	697.6
XH2	1×10^7	10	7	2.4	42	1.679	0.071	1913.6
XH3	1×10^7	10	7	0.95	42	1.007	0.040	5712
XH4	1×10^7	10	7	1.3	42	1.596	nd	226.8
XH5	1×10^7	10	7	1.1	42	0.812	nd	4536
XI0	1×10^7	10	10	6.8	63	0.257	nd	1240
XI1	1×10^7	10	10	5.7	63	0.358	nd	1800
XI2	1×10^7	10	8	15.6	63	0.793	nd	103
XI3	1×10^7	10	10	2.7	63	0.251	nd	936
XI4	1×10^7	10	7	26.9	63	0.288	nd	1360
XI5	1×10^7	10	7	0.89	63	0.330	nd	1608

PLASMA P24 ANTIGEN LEVELS OF PATIENTS INVOLVED IN STUDY

Walter-Reed Stage	p24 Antigen pg/ml	Subsequent p24 tests
1	nonreactive	
1	37	
1	42	
1	nonreactive	
1	nonreactive	
1	nonreactive	
1	nonreactive	
1	31	
1K	nonreactive	
2	nonreactive	
2	nonreative	
2	nonreactive	
2	nonreactive	
2	414	
2	nonreactive	
2	nonreactive	
2	nonreactive	
2	nonreactive	nonreactive
2	nonreactive	
2	nonreactive	
2	nonreactive	
2	65.4	
2	nonreactive	nonreactive
3	33	57
3	46	
3	44	67
3	38	
3	nonreactive	nonreactive
3	nonreactive	
3	53	98;179

PLASMA p24 ANTIGEN LEVELS OF PATIENTS INVOLVED IN STUDY

Walter-Reed Stage	p24 Antigen pg/ml	Subsequent P24 tests
3	30	43
3	nonreactive	
3	nonreactive	
3	11.5	
3-4	31	
3-4	nonreactive	nonreactive
3-4	68	
3-4	nonreactive	
3-5	nonreactive	
4	nonreactive	nonreactive
4	14	
4-5	56	
4-5	66	
4-5	nonreactive	
5	55	
5	7.5	
5	11.5	
5	nonreactive	
5	nonreactive	
5	nonreactive	
5	nonreactive	
5	nonreactive	
5	nonreactive	
5	66	
5	2	
5	nonreactive	

Medical Center
(Atlanta)

1670 Clairmont Road
Decatur GA 30033

Veterans
Administration

In Reply Refer To

February 10, 1989

Dr. Neal Wetherall
Assoc. Prof. of Pathology
Vanderbilt Univ. Med. Ctr.
Nashville, Tenn 37232-2501

Re: Grant proposal

Dear Neal:

This letter is to assure you of our collaboration regarding the evaluation of the compounds produced in our laboratory as potential anti-human immunodeficiency virus type 1 agents in your animal model. As you know, I participate in two of the NIH's AIDS National Collaborative Drug Discovery Groups and we have a large group of organic chemist at the VA/Emory, Georgia State University, and the University of Georgia, Athens who have considerable experience in synthesizing nucleosides and other antiviral heterocycles.

I would be interested in providing you with some of the known anti-HIV compounds we have developed in our laboratory, including CS-87 (AzddU or 3'-azido-2',3'-dideoxyuridine) for evaluation in your nude mouse model (see attached papers). It would be important to first establish that AZT is effective in your model. We have developed pharmacokinetic data in mice with both AZT and CS-87 and these data would be made available to you in order to optimize dosing and scheduling.

I wish you good luck with your application.

Sincerely yours,

Raymond F. Schinazi, Ph.D.
Associate Professor of Pediatrics (Emory Univ.)
and Research Scientist (VAMC)

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORT DETRICK, FREDERICK, MARYLAND 21701-5011

February 23, 1989

REPLY TO
ATTENTION OF:

Deputy for Science

Dr. Neal T. Wetherall
Assistant Professor of Pathology
Department of Pathology
School of Medicine
Vanderbilt University
Nashville, Tennessee 37232-2561

Dear Dr. Wetherall:

I have arranged to have sent to you two drugs for testing in your mouse model, AZT (005411) and AVS-5027. Both drugs should be tested at 50 and 100 mg/kg. These are the doses we found active in the Rauscher leukemia virus system. Since you prefer to look for prophylaxis, I suggest day 1 initiation of treatment (intraperitoneal) and given daily until day 11 (with sacrifice on day 12).

Since these are nude mice and 13-15 grams at the beginning of the experiment, I envision some body weight loss from the treatment.

If you have question during any time of the experiment, please let me know.

Looking forward to a productive collaboration.

Sincerely yours,

Michael A. Chirigos,
Ph.D., D.Sc.
Deputy for Science

28th ICAAC, Los Angeles, Calif.

Official Abstract Form

Instructions: Complete this form and submit it for receipt by 29 April 1988. *Only this original form is acceptable (no photocopies).* Additional forms are available from the ASM Meetings Department. Refer to the sample abstract (page vii) for style. Type the title (initial capitals only first, then list all authors (all capital letters), with an asterisk for the person delivering the paper; and then list institutions and short addresses (do not give departments, divisions, buildings, etc.). See "Preparation of Abstracts" for additional required enclosures. *Note: Any poorly prepared abstract unsuitable for direct reproduction will be retyped, and the author will be charged $20.*

Replication of HIV-1 in Nude Mice. NEAL T. WETHERALL* and ANDREA EIRING. Vanderbilt University School of Medicine, Nashville, TN.

Based upon the replicative cycle and unique genome of the Human Immunodeficiency Virus Type 1 (HIV-1), several therapeutic approaches for treatment of infection have been proposed. Many of these involve agents which have demonstrated activity *in vitro*, however, the efficacy of these antivirals can only be assessed through the use of a small animal model which propagates the HIV-1 *in vivo*. One such model of great potential is the utilization of the nude mouse xenotransplanted with a HIV-1 permissive human cell line. Nude mice were exposed to ^{137}Cs irradiation and inoculated with .5 or 1 X 107 human CD4+ lymphoma cells. When a palpable mass was detected, an additional inoculum of HIV-1 infected cells was delivered proximal to the mass. Ten days later, the animals were sacrificed and the tumors were harvested. Non-HIV-1 infected animals were used as controls. Southern blots of the extracted DNA were hybridized with a Bam HI-SSt 1 3'-orf fragment of pBH1OR3, a DNA probe of genomic HIV. Bands at ~ 9.3 Kb and 3.8 Kb were detected. Northern blots of total RNA similarly probed detected ~ 9.3 Kb HIV genomic, 4.3 Kb env, and 1.0 Kb tat-III HIV-1 mRNA. Polyclonal sheep antibodies to viral p24 and gp 120 antigens were also detected in tumor tissue by immunohistochemical localization. These results demonstrate that the HIV-1 can proliferate in a murine system and provide the basis for development of a unique *in vivo* model for the testing of many types of HIV-1 treatment strategies.

"This work is supported by the U.S. Army Medical Research and Development Command under Contract No. DAMD 17-88-C-8071."

Category [H]

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HIV-1 ANTIGENEMIA IN ATHYMIC "NUDE" MICE

Wetherall, Neal T. and Andrea Eiring.

Department Of Pathology, Vanderbilt University Medical Center, Nashville, TN., U.S.A. 37232-2561.

Objective. Several small animal models of HIV infection have been proposed. We have developed a simple means to support the replication of HIV-1 which can produce large populations of infected mice for therapy screening and host-virus interactions.

Methods. Nude mice (3-4 wks old) were exposed to 600 R's of ^{137}Cs irradiation and inoculated with 1×10^7 HIV-1 infected CEM cells. The animals were followed daily for weight gain and tumor progression. Groups of 6 animals were exsangiunated at 8 intervals over a 9 week period and necropsies were performed.

Results. Plasma p24 antigen was detected at day 3 and rose over 9 weeks (>2200 pg ml). Where possible, plasma antigen was neutralized with human antisera to HIV-1. The infected mice did not exhibit any weight loss, but a highly significant difference was seen in tumor progression when compared to controls. Using immunohistochemistry, sheep raised polyclonal antibodies to viral p24 and gp120 detected HIV proteins within tumor cells and mouse splenic macrophages. Electron microscopy revealed rare intracellular lentivirus particles in the CEM cells.

Conclusion. These findings suggest that the nude mouse transplanted with a well characterized HIV permissive cell line should be useful for many immunological and antiviral studies.

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A MOUSE MODEL FOR INVESTIGATING HIV-1 REPLICATION

Graham, Barney and Wetherall, N. Vanderbilt University School of Medicine, Nashville, TN, USA.

Objective. There is great need for a small animal model of HIV-1 infection. We have developed a system in mice to evaluate the effects of chemotherapy and passive antibody on HIV-1 replication.
Methods. An immunomodulation regimen was devised to allow the growth of human CD4+ cell lines CEM (lymphocytic) and U937 (monocytic) in BALB/c mice. Female, pathogen free, retired breeders underwent sublethal irradiation and depletion of L3T4+ lymphocytes utilizing the monoclonal antibody GK1.5. Mice were then inoculated with 2 X 10^7 CEM cells or U937 cells subcutaneously.
Results. By day 8 a subcutaneous plaque could be felt and by day 14 visible tumors were present. Both cell lines formed tumors that grew exponentially. If maintenance GK1.5 was administered tumor growth continued, but without maintenance tumor growth plateaued during week 3 and then regressed. Subcutaneous tumors were infected by inoculation with CEM cells chronically infected with HTLV-IIIB which resulted in HIV-1 antigenemia in mice with both CEM and U937 tumors. Mice remained active and appeared healthy throughout the experiment.
Conclusions. When standardized this system should provide an inexpensive approach to assess anti-HIV therapeutic and prophylactic agents in vivo. The approach could also be adapted to other viral systems in which small animal models are unavailable, and has potential application in many other fields of biomedical investigation.

This work is supported by the U.S. Army Medical Research and Development Command under contract No. DAMD17-88-C-8071.

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